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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Doutor Jonathan Wilson, Investigador Auxiliar no CIIMAR e do Doutor Victor Quintino, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho à minha família pelo incansável apoio, à Filipa, à Inês, à Odete e à Sofia pela paciência e me ter acolhido tão bem. À Joana porque sem ela este trabalho não teria sido possível. Especialmente dedico este trabalho ao Doutor Jonathan Wilson por toda a ajuda que me tem prestado ao longo dos anos. E por fim, mas não menos importante, a todos os meus amigos, cuja lista seria demasiado extensa para enumerar, não sei o que teria acontecido sem vós.

o júri

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palavras-chave glutaminase, glutamina sintetase, glutamato deshidrogenase, amónia, volatilização

resumo

Muitos peixes conseguem sobreviver a elevados níveis de amónia ambiental; entre eles está o *Misgurnus anguillicaudatus*, este peixe tem também a capacidade invulgar de volatilizar a amónia. Uma dos métodos de tolerar níveis elevados de amónia é através da regulação do metabolismo da glutamina. As reacções de catabólise e anabólise deste metabolismo são catalizadas pela glutaminase (Gase), glutamina sintetase (GSase) e glutamato deshidrogenase (GDH) e permitem o consumo ou síntese de amónia. Estas reacções metabólicas poderão estar à volatilização da amónia no intestino do *Misgurnus anguillicaudatus*. O objectivo deste trabalho consistia em caracterizar a expressão dos três genes e do seu possível envolvimento na tolerância e volatilização da amónia. A expressão genética foi analisada no decurso de duas experiências. Numa primeira vários tecidos (cérebro, guelra, rim, fígado, e três partes do intestino), de animais controlo, seriam analisados de forma a determinar uma linha base da expressão dos genes. Na segunda experiência os níveis de expressão genética, para os três genes, em duas porções do intestino (intestino anterior e posterior), de animais expostos a elevados níveis de amónia ambiental, seriam quantificados ao longo do tempo para poder determinar possíveis alterações na expressão. Adicionalmente conduzir-se-ia a análise das sequências genéticas e comparação filogenética das sequências obtidas no decurso do trabalho. Os resultados da sequenciação mostraram elevada identidade entre as sequências do *Misgurnus anguillicaudatus* e os seus homólogos noutras espécies, mas na maior parte dos casos também havia distanciação evolutiva entre as sequências das espécies. Os resultados da expressão genética nos vários tecidos mostraram níveis significativamente elevados para a expressão da Gase e GSase no cérebro, estando de acordo com a importância destas enzimas na reciclagem do glutamato e na eliminação de amónia neste órgão. A expressão dos genes no intestino em resposta à exposição à amónia ao longo do tempo n mostraram alterações significativas, isto sugere que o metabolismo da glutamina não é importante para o processo de volatilização ou que regulação não se dá ao nível da transcrição.

keywords glutaminase, glutamine synthetase, glutamate dehydrogenase, ammonia, volatilization

abstract

Many fishes are able to cope with high levels of environmental ammonia; among them is the oriental weatherloach (*Misgurnus anguillicaudatus*), which also has the unusual ability to volatilize ammonia. One proposed method of ammonia tolerance in this species is through the regulation of the glutamine metabolism. Glutaminase (Gase), glutamine synthetase (GSase) and glutamate dehydrogenase (GDH) catalyze reaction in which catabolysis and anabolysis lead to the release or consumption of ammonia, and can possibly be associated with ammonia volatilization in the gut of the weatherloach. This work intended to characterize the expression of these three genes and their possible involvement in ammonia tolerance and ammonia volatilization in the gut of the weatherloach. In order to determine the gene expression levels two experiments were analyzed separately. In one, several tissue samples (brain, gill, liver, kidney and three portions of gut) from control animals would be analyzed in order to determine baseline expression levels of the three genes. In the second experiment gene expression levels for the three genes in two sections of the gut (foregut and hindgut) from animals that had been exposed to high environmental ammonia over time would be analyzed in order to determine possible changes in gene expression. Additionally gene sequence analysis and phylogenic comparison would be carried out on the gene sequences obtained during the work. Sequencing results showed high identity between weatherloach and their homologues in other species for each gene, but in most cases with significant evolutionary branching from other species. The results for gene expression in several tissues showed significantly elevated levels of gene expression for Gase and GSase in the brain, which correlates with the importance of these enzymes in glutamate recycling and ammonia detoxification in this organ. The results for gene expression in gut in response to ammonia exposure over time showed no significant changes suggesting that glutamine metabolism is not important in ammonia volatilization or that regulation is not at the transcriptional level.

INTRODUCTION

The oriental weatherloach, *Misgurnus anguillicaudatus*, is a freshwater teleost, of the loach family (Cobitidae), which inhabits still or slow moving, shallow water bodies with muddy bottoms, such as swamps and rice paddies in Asia ranging from China to Myanmar (www.fishbase.org). The name weatherloach is derived from the ability they have to detect changes in barometric pressure before a storm making them more active even during the daylight even though they are mainly nocturnal. The weatherloach has developed its intestine as an accessory air-breathing organ and air-breathes by gulping air at the water's surface and releasing bubbles from its vent which has enabled it to survive periods of aquatic hypoxia. The regions these fish inhabit are also prone to droughts; during which they actively bury themselves in the soft muddy bottoms. The muddy bottom helps to maintain its body moist; and by using its gut as an accessory breathing organ it can air-breath to survive prolonged droughts (McMahon and Burggren, 1987). This unique use of its gut also allows the weatherloach to volatilize excess ammonia (Tsui *et al*, 2002). Ammonia excretion into the water is impeded during periods of drought because the gills are deprived of a water flow to carry away the excreted ammonia. The volatilization process, as such, allows the weatherloach to eliminate ammonia while by passing the need for a water flow through the gills. In addition to the afore mentioned volatilization, the weatherloach is also extremely ammonia tolerant, which allows it to live in extremely poor quality water with high ammonia levels that would be lethal to most other fishes.

Ammonia toxicity

High ammonia concentrations can be an issue most notably for weatherloaches that live in rice paddies, which are heavily fertilized with both natural fertilizers and ammonium sulphate. Thus they must be capable of tolerating high levels of ammonia. The weatherloach is a case where several strategies for ammonia tolerance and excretion can be found in the same species (Tsui *et al*, 2004).

Before discussing the evolutional adaptations the weatherloach has acquired to both tolerate and excrete ammonia, the question of why ammonia is toxic to fish must be posed. Ammonia is found in the environment as a result of animal waste, the decomposition of organic material, release of fertilizers, industrial emissions and volcanic activity. Volatilized ammonia from the earth's surface and atmospheric ammonia are destroyed by photolytic reactions, though some may return in rainwater most of the ammonia present in the atmosphere poses no serious problem, aquatic exposure to ammonia is a problem however (Randall and Tsui, 2002).

NH_3 is a Brønsted base, which means that it is a compound that accepts protons. In aqueous solution, ammonia has two species, the gaseous form NH_3 and the cation NH_4^+ (ammonium), and is a rather weak base (K_b=1.7x10^{-5}). The equilibrium distribution of the $NH_3/NH_4^+$is strongly determined by pH but very less affected by pressure and temperature. At neutral pH almost all of the ammonia will be in its cationic form (NH_4; >99%), but as pH raises so does the concentration of NH_3 in solution.

Biological membranes (lipid bilayers) have a low permeability to NH_4^+; however; they are relatively more permeable to NH_3. Upon entering the body NH_3 reacts with free protons forming NH_4^+, though NH_3 by itself does cause an alkalization NH_4^+ is considered the major toxic element for fish because it interferes with ion balance and membrane potential (Ip *et al*, 2001). The high amounts of ammonia in heavily fertilized water bodies increase the environmental concentration of NH_3 resulting in a net uptake of ammonia and consequently ammonia intoxication.

During aerial exposure fish cannot exchange gases efficiently through the gill as they normally would, which leads to a build up in internal ammonia as well. This in turn leads to the necessity to eliminate or detoxify ammonia by other means.

Ammonia has been found to cause various behavioural and physiological changes detrimental to both the animal's health and consequently survival. Gill hyperplasia (the proliferation of cells within an organ or tissue beyond that which is ordinarily seen) can result from chronic ammonia exposure (Ip *et al*, 2001). Elevated water ammonia levels have been shown to inhibit both feeding and growth (Hampson, 1976; Alderson, 1979) and reduce swimming capability (Shingles *et al*, 2001; Wicks *et al*, 2002). The reduction in

swimming capability found by Shingles *et al* (2001) was attributed to the depolarization of the muscles due to the increase in ion levels, which is another aspect of ammonia's effect on the body. As mentioned ammonium cannot easily cross biological membranes, but it is capable of substituting K^+ in ion exchanges and thus altering membrane potential (Randall and Tsui, 2002). Ammonia has also been found to interfere with the tricarboxylic acid cycle (Arillo *et al*, 1981), stimulating glycolysis in fish muscle and affecting many other metabolic pathways (Ip *et al*, 2001).

One of the main mechanisms of ammonia toxicity is its effect on the central nervous system. Ammonia crosses the blood brain barrier, and as mentioned before, can alter membrane potential. The alterations in membrane potential and conductivity lead to abnormal and excessive activation of NMDA glutamate receptors that eventually lead to convulsions and ultimately death. The use of NMDA antagonist blockers have a similar effect on mammals and fish protecting the animal from the subsequent ammonia related death (Randall and Tsui, 2002).

Protection from ammonia

Ammonia is toxic to the very beings that produce it and thus its build-up in the body is not solely due to uptake from water being a by-product of several metabolic pathways, most notably of protein catabolism. There are three major forms of eliminating nitrogenous waste, the excretion of urea by ureotelic animals, this includes mammals and other terrestrial and amphibian species and also some fish (Wright 1995). There is also the excretion of uric acid most notably by birds but also in reptiles, they are therefore called uricotelic. The other method is that which the vast majority of teleost fishes use, the excretion of ammonia directly, therefore fish are called ammonotelic (Anderson, 2001).

Ureotelic animals maintain ammonia levels in the blood low, the process requires energy to convert ammonia into a less toxic product that can be stored for future elimination, urea. In order to convert ammonia to urea most animals use the ornithine urea cycle in the liver. Ammonia resulting from amino acid catabolism is converted to

carbamoyl phosphate, by carbamoyl-phosphate synthetase I, which then enters the urea cycle (Anderson, 2001). This cycle occurs both inside and outside of the mitochondria, eventually arginine is converted to ornithine and urea.

The need to store a nitrogenous waste like urea has to do with one of the major problems of life above water, the need to conserve water, since drinkable water sources may not always be available. Additionally, in birds the need to store aqueous solutions of urea adds weight which is detrimental to flight; uric acid is eliminated in the feces which helps to retain water. It is necessary to actively form a less toxic nitrogenous waste which can be stored until excretion is possible. Urea and uric acid can be stored and concentrated in body fluids in greater concentrations with no toxic effects and their excretion requires, respectively, 10 and 50 times less water than ammonia (Wright, 1995). Most elasmobranches also produce urea; however, they are denominated ureoosmotic since they use urea in their osmoregulation (Anderson, 2001).

Furthermore, fish by not needing to deal with water conservation, since it is readily available, can excrete ammonia during normal gaseous exchange across the gill. Though most teleost fish are, for the large part ammonotelic, most do excrete a smaller portion of nitrogenous waste as urea (10%; Anderson, 2001). Urea in teleost derives from three possible sources uricolysis, catabolism of dietary arginine and/or the ornithine urea cycle (OUC) although this is rarely observed in adult teleost fishes (Wright, 1995). In ammonotelic teleosts urea is generally derived from uricolysis (Mommsen and Walsh, 1989). For uricolysis to occur the catabolism of purine nucleotides ultimately leads the production of uric acid, which is subsequently converted to urea. However, in teleost fishes urea is not part of ammonia detoxification since they lack the OUC but instead results, as mentioned, from uricolysis and arginolysis.

Urea, as mentioned, is less toxic than ammonia and serves its purpose for both excretion of nitrogenous waste and osmoregulation. Nevertheless ammonia also serves a purpose despite its toxicity. It is believed to be important in intercellular buffering. Though most of the ammonia excretion occurs through the gills and skin, synthesis and excretion in the kidneys, for example, also has an important role in regulating chronic metabolic acidosis (Wood *et al*, 1999). In mammalian kidney through glutamine and other

amino acid catabolism, by enzymes like glutaminase (Gase), NH_4^+ and HCO_3^- are producted. NH_4^+ is excreted and HCO_3^- is retained by the kidney and returns to the blood stream resulting in a net acid flux. Most of the research on this topic has been done in mammals, but there is evidence of this happening in fish as well (Wright, 1995; Wood *et al*, 1999).

When facultative air-breathing fish are out of water, ammonia excretion is compromised since ammonia excretion through the gills into the ambient water is impaired. Fish that can excrete and store urea still have one way of eliminating ammonia, though ammonia excretion largely occurs across the gills the ability to store a less toxic compound like urea helps avoiding toxic build up of ammonia. The weatherloach, like most other teleost fishes, cannot use this pathway since it does not have the complete complement of OUC enzymes and therefore needs alternatives (Chew *et al*, 2001).

Elevated levels of environmental ammonia impair excretion, which is normally accomplished through the gills, and can even cause an involuntary uptake of ammonia (Ip *et al*, 2001). Different species use different strategies to protect and deal with ammonia, like the afore mentioned use of the urea cycle. Ammonia toxicity can be ameliorated by preventing accumulation. This is achieved by decreasing production, maintaining or enhancing excretion and/or conversion of ammonia to less toxic compounds. Most fish species cannot tolerate high levels of ammonia; while some like the oriental weatherloach can, and are therefore considered ammonia-tolerant. As stated by Tsui *et al* (2004), a notable aspect of the oriental weatherloach is that many of the strategies and adaptations for dealing with ammonia can be found in this unique species.

Proteins and amino acids are the major energy sources for long-term muscular activity. Preventing the complete catabolism of amino acids and/or partial catabolization to alanine can reduce the formation of ammonia while still allowing some energy production. Chew *et al* (2001) has demonstrated that alanine levels in the liver of the oriental weatherloach rose after aerial exposure, which supports the theory that this is one of the methods used for ammonia tolerance.

Ammonia levels in tissues, most notably the brain, are usually kept low in order to maintain normal functionality. In weatherloaches, however, it has been reported that

ammonia levels in muscle and liver are among the highest reported (Tsui *et al*, 2004). The mechanism for this high tolerance of ammonia is unclear; nonetheless it may possibly be related to a relative insensitivity of enzymes to ammonia or a close regulation of internal pH.

As mentioned above ammonia's effect on the central nervous system is one of its most dangerous aspects. NH_4^+ and K^+ are similar in their radii which allow for NH_4^+ passage through K^+ channels which in turn leads to depolarization of excitable tissues in resting states (Tsui *et al*, 2004). As stated the weatherlaoch can tolerate high levels of ammonia in its tissues and apparently do not have problems with membrane depolarization. T.K. Tsui and colleagues (2004) have hypothesized that the weatherloach has very selective K^+ channels that impede the passage of NH_4^+. This allows for the membrane's relative impermeability to NH_4^+ and allows for the maintenance of membrane potential.

The oriental weatherloach has two more strategies which allow for removal of high levels of ammonia. One of those strategies is the formation of glutamine. Glutamine synthesis is a two step process that begins with α-ketoglutarate, one of the products of the tricarboxylic acid (TCA) cycle, which is converted to glutamate in a reaction catalyzed by glutamate dehydrogenase consuming one NH_4^+ in the process. With the subsequent reaction between glutamate and another NH_4^+ ion, catalyzed by glutamine synthetase this process actively removes two NH_4^+ molecules. This process is known as being part of the weatherloach's ammonia detoxification strategy. It has been documented that glutamine levels in muscle, liver and brain rise in response to aerial exposure and therefore ammonia loading (Chew *et al* 2001). The drawback of this process is that for each synthesized molecule of glutamine two molecules of ATP are hydrolyzed. It is suspected that the oriental weatherloach can use this strategy because it remains relatively motionless after burying itself in the mud. By not needing energy for muscle activity it can focus energy toward NH_4^+ removal during later stages of aerial exposure.

One final documented, and rather special strategy *M. anguillicaudatus* uses to rid itself of ammonia is by volatilization of NH_3 through the gut and skin which occurs with exposed to high environmental ammonia levels in water as well as during emmersion

(Tsui *et al*, 2002). The high levels of endogenous ammonia the weatherloach can tolerate may create a gradient that allows for the passage of ammonia through the relatively thin gut wall (Tsui *et al*, 2004). When the weatherloach shallows air, oxygen uptake can occur in the highly modified intestine and $NH_{3,}$ along with CO_2, can be released. These exchanges are possible because of the structure of the weatherloaches gut which has thin walls associated with a heavily vascularised epithelium. Intraepithelial capillaries pass closely to the lumen of the digestive tract allowing for the gas exchange to occur (McMahon & Burggren 1987; Gonçalves *et al*, 2007). The intestine of the weatherloach has three distinct regions with distinct functions; the foregut is glandular and morphologically suited for digestion and nutrient absorption. Foregut and hindgut are separated by a spiral zone, which compacts the remaining undigested material to keep the walls free of material which in turn facilitates gas exchange (McMahon and Burggren, 1987). Recently Gonçalves *et al* (2007) demonstrated that the hind-gut serves a dual function, not only was it involved in gaseous exchange, as morphology suggested but it also retained a role in nutrient uptake.

Ammonia can also be volatilized in a similar way through the skin, in its natural habitat; however, when the loach is buried in the mud, skin volatilization may be impeded from being a site for NH_3 volatilization (Tsui *et al*, 2002).

The problem that resides with volatilization is that, though documented as occurring in weatherloach (Tsui *et al*, 2001), the question as to how ammonia reaches the gut for elimination has not been addressed. Ammonia levels in weatherloach plasma, liver and muscle are very high (Chew *et al*, 2001; Tsui *et al*, 2002). Thus, one proposed possibility is that ammonia travels through the blood stream and transverses the cell membrane directly, be it actively or passively and is excreted into the lumen. However in mammals, it is well documented that enterocytes preferentially use glutamine as an oxidative fuel and in fish glutamine has also be found to be important to these cells (Yang *et al*, 2000; Yan and Qui-Zhou, 2006). Another proposed theory is that rather than ammonia circulating freely through the blood stream the ammonia produced in the liver and muscles is converted to glutamine, travels through the blood stream and upon reaching volatilization sites is catabolised releasing ammonia similar to the process

observed in kidney during metabolic acidosis described earlier (Wood *et al*, 1999). Both of these possibilities allow for ammonia volatilization to occur (Tsui *et al*, 2004).

Glutamine metabolism

Glutamine has been shown to have many roles: as an energy source, an essential component in protein structure and nucleic acid synthesis, storage of nitrogenous waste and as a transporter of nitrogen in the body, the later in mammals (Ip *et al*, 2001).

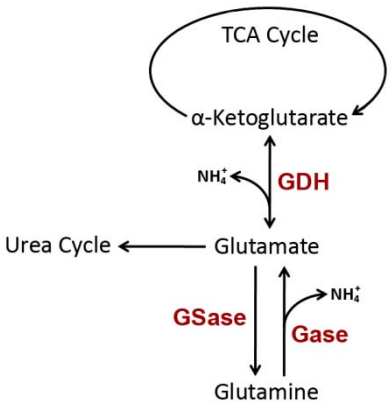

Figure 1 – Diagram of the glutamine metabolism and the enzymes involved, glutaminase (Gase), glutamate dehydrogenase (GDH), and Glutamine synthetase (GSase).

Amino acids serve as important energy sources for fish, glutamine in particular, along with glutamate and proline, were shown to be oxidized in fish mitochondria and serve as an energy source in muscle (Chamberlin *et al*, 1991). The initial breakdown of glutamine is catalyzed by glutaminase (Gase), which converts glutamine to glutamate. Glutamine can be completely oxidized into acetyl-CoA, entering the TCA cycle with oxaloacetate which would include the deamination of glutamate, by glutamate dehydrogenase (GDH), into α-ketoglutarate. The end result is energy production. One drawback of using glutamine as an energy source is the release of ammonia in both of the steps mentioned above.

However through reversed pathway resulting in glutamine synthesis it is possible to actively remove ammonia. As mentioned above, α-ketoglutarate can be converted to glutamine; this step requires two NH_4^+ ions for the reaction to occur which are actively removed. The two step process is catalyzed by glutamate dehydrogenase, converting α-ketoglutarate and ammonia to glutamate, and glutamine synthetase (GSase), converting glutamate to glutamine, also requiring ammonia. The one drawback of this process is the expenditure of 2 ATP molecules.

Each of the enzymes mentioned above serve different purposes in the metabolic pathway and their expression levels vary accordingly. For example the primary mechanism for amino acid catabolism in fish liver is the conversion to glutamate and subsequent deamination by glutamate dehydrogenase. Glutamine synthetase and glutaminase also have several important roles. In the gill it was found that glutamine synthesis may provide a control over the release NH_4^+ ions (Mommsen, 1984). In fish posterior region of the gut, glutamine synthetase has been proposed to minimize the backflux of ammonia from the lumen into the blood by trapping it as glutamine (Mommsen *et al*, 2003). Glutamine synthetase has extremely important roles in the brain: controlling ammonia levels, by synthesizing glutamine and actively removing ammonia, which as mentioned alters membrane potential and also has an important role in glutamate recycling. Also, glutamate is a neurotransmitter released into the synapse that excites the NMDA glutamate receptors; it can be reabsorbed by the cell or converted to glutamine by glutamine synthetase and then absorbed. Glutamine can then be reconverted to glutamate thus completing the recycling (Wood *et al*, 1999) process.

Also, and as mentioned above, ammonia synthesis in mammalian kidney serves a purpose in compensating for chronic metabolic acidosis. Glutamine is transported to the kidney where glutaminase and glutamate dehydrogenase are involved in the process of ammoniagenesis liberating NH_4^+ and HCO_3^-. NH_4^+, a weak acid, can then be excreted via the urine to compensate for the chronic metabolic acidosis. Fish kidney seems to behave in a similar manner to the mammalian kidney (Wood *et al*, 1999). Though the kidney of some marine elasmobranchs exhibits little flexibility or capacity for dealing with acid-base regulation (Maren *et al*, 1992; Swenson *et al*, 1994). Wood *et al* (1999) suggests that the kidney function of some freshwater teleosts has similar mechanisms and comparable flexibility to that of the mammalian kidney.

In summary, glutamine and other amino acids, catabolism and anabolism for various purposes require the activity of the three enzymes mentioned above and the focal point of this investigative work: glutaminase, glutamine synthetase and glutamate dehydrogenase.

Objectives

Due to the environmental conditions the oriental weatherloach, *Misgurnus anguillicaudatus* must endure in the wild it has developed a high tolerance to environmental ammonia by employing various strategies. This investigative work intends to shed a light on one in particular, involving glutamine metabolism. To accomplish this the levels of gene expression of three known enzymes of the glutamine metabolism, glutamate dehydrogenase, glutaminase and glutamine synthetase, by employing reverse transcription polymerase chain reaction (RTPCR) using a semi-quantitative approach

Two different experiments will be conducted, first to study tissue distribution (brain, gill, kidney, liver, foregut, mid-gut and hind-gut) of the enzymes' transcripts under basal conditions. A second analysis of samples will attempt to determine changes in the gene expression of all three enzymes in the foregut and hindgut regions of the intestine under basal conditions and during ammonia loading in order to test the hypothesis that glutamine may be important in ammoniagenesis at the site of ammonia volatilization in the loach gut. Glutamine synthetase transcript levels were also measured in these tissues since this enzyme has been proposed to prevent the backflux of luminal ammonia by trapping it as glutamine (Mommesen *et al*, 2003) in addition phylogenetic analysis will be carried out on loach glutaminase, glutamine synthetase and glutamate dehydrogenase. In the case of glutaminase a 3' Rapid Amplification cDNA Ends (RACE) will be carried out in order to acquire a greater proportion of the gene sequence for analysis.

MATERIAL AND METHODS

Animals

Adult weatherloach were purchased from the main wet market in Yuen Long Hong Kong and stabilized at City University of Hong Kong before transport to CIMAR (Centro Interdisciplinar de Investigação Marinha e Ambiental) by air freight. Fish were transported with minimal water. Upon arrival fish were maintained in 100 L glass aquaria containing dechlorinated Porto city tap water (Na^+ 0.5mM, hardness 50mg/L $CaCO_3$, pH 8), with constant aeration at 19^oC, under natural light conditions. Once a week, 20% of the water was changed. During this period the fish were fed daily *ad libitum* with commercial fish food (Granured, Sera Gmb, Germany), prior to the experiments fish were starved for 48 hours.

Experiments:

- Time course ammonia exposure

Weatherloaches, weighing 9.11±3.86 g and measuring 11.08±1.46 cm, Mean±SD (Standard Deviation), were exposed to high environmental ammonia (HEA), 6.3 mM NH_4Cl at pH 8 (0.244 mM UIA), in a time course experiment. Five animals were sampled at 0, 1, 6 and 24h of HEA or from a parallel control exposure.

- Chronic ammonia exposure

Weatherloaches, weighting 8.47±4.88 g and measuring 11.05±1.78 cm, Mean±SD, were used for chronic ammonia exposure (CE). Five fish exposed to environmental ammonia were exposed to a level of 2.9 mM NH_4Cl at pH 7.6 and 19^oC (0.042 mM UIA), for a long period (more than two months), to simulate natural habitat conditions.

Sampling

Fish were euthanized by an overdose of neutralized MS222 (1:5000 (w/v) Aquapharm, UK). Fish were measured to the nearest millimetre and weighed to the nearest milligram, for condition factor calculation. No differences in condition factor (K) were found between groups in time course and in *in vitro* ammonia flux experiments. The caudal peduncle was severed and blood was collected in heparinised capillaries, centrifuged, hematocrit measured and plasma immediately frozen in liquid nitrogen. Foregut and hindgut tissues were excised and immediately frozen in liquid nitrogen.

Foregut, midgut, hindgut, gill, kidney, liver and brain of control animals were collected from a separate set of animals for gene tissue distribution analysis.

All samples were stored at -80^{o}C, until analysed. The tissues samples used and analyzed were collected during a previous experiment done in our lab.

Primer selection and design

Primers were synthesized by STABvida (Oeiras, Portugal). Primer sequences for glutaminase (Gase), glutamate dehydrogenase (GDH) and glutamine synthetase (GSase) were predicted using the online software Primer 3 (http://frodo.wi.mit.edu). Primer design was a two step process, first consensus primers were designed for a 500-600bp product which was then sequenced and its identity confirmed. From this sequence, loach specific primers were designed for a 200-300bp product for semi-quantitative PCR analysis; all primer pairs are shown in table 1. The consensus primer sequences for Gase had already been designed by our lab using multiple alignments of Gase sequences, GSase consensus primers were obtained from Mommsen *et al* (2003).

The GDH consensus primers were designed by me for this work using the following parameters, on Primer3, T_m (melting temperature) range was 56-62^{o}C with a 60^{o}C optimum, primer length between 18-25bp with a 20bp optimum and 45-55% GC content. As mentioned product size for GDH was initially 500-600bp and the template used for selection was glutamate dehydrogenase 1 sequence for zebrafish (ensembl.org id

ENSDARG00000002414), since it was the closest phylogenetically to weatherloach, from the fishes in the Ensembl database to generate a list of potential primers. Multiple alignments (MultAlin; http://bioinfo.genotoul.fr/multalin/ multalin.html) with fugu (*Takifugu rubripes*; ensembl.org id ENSTRUG00000000321), medaka (*Oryzias latipes*; ensembl.org id ENSORL G00000010426), stickleback (*Gasterosteus aculeatus*; ensembl.org id ENSGACT00000007967) and pufferfish (*Tetraodon nigroviridis*; ensembl.org id ENSTNIG00000005331) from the Ensembl database as well as rainbow trout (*Oncorhynchus mykiss*; genbank id AF427342) and Atlantic salmon (*Salmo salar*; genbank id BT044654) sequences from the GenBank database were used to identify conserved regions in the gene sequence for selecting a consensus primer pair. The sequences were then analyzed on the BLAST (Basic Local Alignment Search Tool) software of the NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov) using the GenBank database. The tools used were nucleotide BLAST (BLASTn) and primer-BLAST which checked the database for the sequences of the primers, the primer pairs that returned the most results with the best sequence similarity scores (E value) corresponding to the intended genes selected.

For the design of loach specific primers the sequences used were the ones amplified in our lab by PCR using the previously mentioned primers. Sequencing results were cross-checked on the GenBank database to confirm amplification of the correct genes. This was done by both using BLASTn and also BLASTx (searches the protein database using a nucleotide sequence) if the results corresponded to known sequences for the targeted genes then the correct sequences had been amplified.

In addition to the primers mentioned above β-Actin (actin or Act) was used as a "house-keeping" gene and was originally designed for the Gilthead seabream, *Sparus aurata* (Santos *et al*, 1997), the product of this primer pair has already been sequenced in weatherloach (unpublished). The primer pair for actin was ordered from the Sigma-Aldrich group Proligo (St. Louis, MO, USA).

Table 1- Primer pairs along with primer sequences and the expected amplicon size. Glutamate dehydrogenase (GDH), Glutaminase (Gase), and Glutamine synthetase (GSase) represent the consensus primers. L-GDH, L-Gase and L-GSase represent the primers designed specifically for weatherloach. Act stands for the β-actin primer pair.

	Forward Primers	Reverse Primers	Amplicon Size
GDH	5'- CTTTGGTAATGTGGGTCTGC -3'	5'- TGCACAATGTCCTTTTCAGA -3'	≈600bp
GSase	5'- GAGGGCTCCAACAGCGACAT -3'	5'- CAGCCAGCACCGTTCCAGTT -3'	≈600bp
Gase	5'- GTGGCAGACTACATTCCTCAG -3'	5'- AGTCGTACATGCCGCAGGA -3'	≈600bp
L-GDH	5'- CTGCTGCAAGTGAAAAGCAG -3'	5'- GCCACTCGAAGTAGGAGACG -3'	≈190bp
L-GSase	5'- GGTCCCCAAGGACCCTACTA -3'	5'- GAGCGACCCACAAATGATCT -3'	≈200bp
L-Gase	5'- GCCTGTGATTGGACAGAATC -3'	5'- GAAGATGGCAGGAAATGAAT -3'	≈240bp
Act	5'- GGCCGCGACCTACAGACTAC -3'	5'- ACCGAGGAAGGATGGCTGGAA -3'	≈300bp

RNA Extraction and quantification

RNA extraction and isolation was carried out with the Illustra RNAspin Mini RNA Isolation

Kit from GE Healthcare (Chalfont St. Giles, UK), following the protocol in the product booklet. Tissues were homogenized by sonication at 40% intensity for two to three 2 second bursts (Sonics & Materials Inc, CT, USA) and further homogenized by carefully passing the homogenate through a 23 gauge needle and syringe 7 to 8 times. RNase AWAY (Molecular BioProducts, San Diego, CA, USA) was used on all dissection material, as well as on the sonicator horn, for tissue sample collection and preparation in order to remove any contamination with RNases thus minimizing RNA degradation. The tissue samples for the tissue distribution had been previously collected and stored in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) and immediately frozen in liquid nitrogen, they were then stored at -20^{o}C. DNase treatment was performed on column, in order to digest any DNA present on the silica membrane of the RNAspin mini column (provided in the kit), was accomplished with 185U (per extraction) DNase I from GE Healthcare. The only steps taken that differed from the GE Healthcare protocol were the use of 50% ethanol rather than 70% ethanol suggested in the protocol and the elution with 50µL of Sigma-Aldrich molecular biology grade water and the re-centrifugation of the final flow-through through

the column for 1 minute in order to obtain a higher concentration of extracted RNA. RNA samples were placed on ice once final elution was completed and stored at -80^{o}C.

RNA concentration was determined by use of a Genova spectrophotometer (Jenway, Essex, England) 1µL of extracted RNA solution was added to 150µL of MilliQ water for the measurements/quantifications. The spectrophotometer automatically calculated the ratio of the optic absorptions for wavelengths 260 (which is the absorption wavelength of nucleic acids) and 280nm (the absorption wavelength of proteins), this would indicate any possible contamination with proteins; ideally the ratio would be between 1.8 and 2.2 which would indicate minimal protein in solution. The spectrophotometer also calculated the concentration of nucleic acids in solution. Genomic DNA contamination was found to be minimal by analysis of a 1.2% agarose formaldehyde gel with 0.0001µg/ml of ethidium bromide (EtBr), for staining, added to the gel. The images were acquired with a Kodak EDAS 290 gel documentation system.

cDNA synthesis

RNA extraction products were reverse transcribed using the iScript cDNA Synthesis Kit from BioRad (Hercules, CA, USA). Reagents used were provided in the kit and reaction volumes for a total of 20µL reaction volume, 4µL of 5xreaction mix, 1µL reverse transcriptase RNase H+, the equivalent of 1µg of RNA and the remaining volume of nuclease free water. Thermal cycler parameters used were 25^{o}C for 5 minutes, 42^{o}C for 30 minutes and 85^{o}C for another 5 minutes. The cDNA was then stored at -20^{o}C.

PCR optimization

The optimum condition for PCR reactions for each of the loach specific primer pairs was determined in much the same way as described by Marone *et al* (2001). Five $MgCl_2$ concentrations in the reaction mix were tested; 1, 1.5, 2, 3, and 5mM in order to determine which produced the best results. Three concentrations were chosen, 2mM for Actin, 1.5mM for L-GDH and 1mM for both L-Gase and L-GSase.

The optimum annealing temperature was determined by using a temperature gradient in our thermalcycler ranging from 53°C to 64°C. For all primer pairs 60°C was chosen as giving the best results.

Optimum cycle number was also determined in order to produce the strongest possible bands while still ending the PCR reaction in the exponential phase of the reaction. This would avoid quantifying bands in the plateau phase of the reaction where all results would be maximized and little to no differences between bands would be found. A sample was taken, by removing a reaction tube and allowing it to finish elongation on a hot block for 5min, every two cycles after the 20th an up to the 36th cycle. The optimum cycle number for actin was 24 cycles and 30 cycles for the other three genes. See table 2 for a summary of optimum parameters for each gene.

Table 2- Summary of optimum conditions for weatherloach specific and Actin primer pairs. Optimum values for $MgCl_2$ concentration, annealing temperature and cycle number for L-GDH (glutamate dehydrogenase), L-Gase (glutaminase), L-GSase (glutamine synthetase) and Actin (β-actin)

Primer pair	[$MgCl_2$]	Annealing Temp	Cycle number
L-GDH	1,5mM	60°C	30
L-Gase	1mM	60°C	30
L-GSase	1mM	60°C	30
Actin	2mM	60°C	24

RT-PCR

For a total PCR reaction volume of 20µL, 0.8µL of synthesized cDNA was amplified by 0.8 units of DyNAzyme II DNA polymerase (Finnzymes, Oy, Finland). The reaction mix for the PCR reaction was completed with final concentrations of 1x reaction buffer (10x stock, provided with the Taq), 0.8mM dNTPs (40mM stock solution), and 0.4mM of the primer pair (10mM stock solution). The volume of $MgCl_2$ added to the mix varied with the desired concentration in the final reaction mix (1, 1.5 or 2mM) however the stock solution

used was the 50mM solution also provided by the Taq supplier. Sigma-Aldrich molecular biology grade water was used to complete the 20µL reaction volume.

Table 3- Layout of the thermalcycler program. The box encompassing the 2nd, 3rd and 4th steps represents the cycled steps. Cycle number varied according to the optimum cycle number for each of the primer pairs (30 cycles for L-Gase, L-GSase and L-GDH; 24 cycles for actin). Elongation time in each cycle also varied according to the amplicon size, it was considered to be roughly 1 minute per kb.

Steps	Temp	Time	Phase	
1st	94°C	2min	Denaturing	
2nd	94°C	30sec	Denaturing	24-30 cycles
3rd	60°C	30sec	Annealing	
4th	72°C	15-30sec	Elongation	
5th	72°C	5min	Elongation	

PCR reactions were carried out on a MJ Mini Personal Thermal Cycler (BioRad) according to the following steps (see table 3): 1st 94°C for 2 minutes, 2nd 94°C for 30 seconds, 3rd 60°C for 30 seconds (this corresponded to the optimum annealing temperature though other temperatures were used, this is discussed further on), 4th 72°C for 15 to 30 seconds (elongation time depended on product size I considered this to be roughly 1 minutes per kb), the 5th and final step for reaction to occur was 72°C for an additional 5 minutes in order to finish elongation. Steps 2 to 4 were repeated for 24 to 30 cycles for quantification, depending on the primer pair and 40 cycles for sequencing. PCR products were then stored at 4°C.

Gel electrophoresis

The PCR products, for all reactions, were loaded onto 2% agarose TBE (89mM Tris/89mM Borate/2,5mM EDTA) gels in TBE buffer. A 100bp DNA ladder molecular weight marker from Bioron (Ludwigshafen, Germany) was included in every gel to confirm or determine the molecular weight of the amplified product. All gels were stained with ethidium bromide (EtBr). Initially the EtBr was added to the gel so that it would have final

concentration of 0.0001µg/ml. In order to have a uniform background in the gels for semiquantitative analysis they were post-stained with an EtBr bath (0.05µg/mL) for 15 to 25 minutes and rinsed in MilliQ water afterwards for 15 minutes.

Image acquisition and Semiquantitative analysis

The RT-PCR protocols described above are considered semiquantitative; the subsequent quantification of the bands was carried out with the SigmaScan Pro 5 software (Systat Software Inc, San Jose, CA, USA). The use of this software allowed for the determination of band area and average intensity, this in turn gave an indirect quantification of the band, which allowed me to infer about gene expression levels without knowing exactly how many copies of the amplified gene there were.

Images of the gels were acquired with a Kodak EDAS 290 gel documentation system and saved as tiff files. The intensity threshold, for image analysis was manually set in order to allow the best possible quantification of the bands. Band intensity is expressed as "Area x Average intensity" of the band. The ratio between gene expression and actin was calculated in order to normalize the data and the ratios are presented as relative changes from control group.

DNA extraction and purification from TAE gels

In order to sequence the amplified PCR products a similar reaction mix as described above was used with only an increase in total volume to 50µL and as mentioned 40 cycles in order to maximize amplified sequences. These PCR products were all loaded onto 1% agarose TAE (40mM Tris/40mM Acetate/1mM EDTA) gels in TAE buffer with 0.0001µg/ml EtBr. Bands were excised under as little UV light as possible, to prevent possible DNA damage, and purification carried out with the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare, Chalfont St. Giles, UK) according to the protocol provided in the user's booklet. The only exception to the protocol was the use of 30µL of the kits elution buffer type 6, rather than the 50µL of elution buffer type 4 mentioned in

the protocol, the flow-through was re-centrifuged through the column in order to obtain a higher yield of recovered DNA. The samples were subsequently sent to STABvida for sequencing.

3' RACE of Gase gene

In order to obtain a greater length of gene sequence for Gase a 3' RACE (Rapid Amplification of cDNA Ends) was done. The method followed was similar to the one described by Hung *et al* (2007). A Superscript III First-strand synthesis system for RT-PCR from Invitrogen (Barcelona, Spain) was used for cDNA synthesis and addition of an adapter primer sequence to the 3' extremity of the cDNA molecules. The protocol for cDNA synthesis was similar to the one provided with the kit, reagents were added accordingly with the only exception being the use of an adapter primer rather than the oligodT primer provided in the kit. The temperatures used during the incubation periods for the synthesis protocol were the main difference between both protocols; the primer ligation period was at 70^{o}C for 10 minutes followed by 5 minutes at 4^{o}C. The reverse transcription was carried out for 2 hours at 37^{o}C.

The adapter primer added a known sequence (GACTCGAGTCGACATCGA) to the 3' poly-T extremity of the cDNA, by using a reverse primer for that sequence and a forward primer, previously used for the rt-PCR (forward primer of the L-Gase pair), this allowed for the amplification of an approximate 1000 bp amplicon.

This amplicon was extracted and purified as described above for insertion into a cloning vector and cloning in *E. coli* so as to obtain a high yield for sequencing. Cloning was carried out with a PCR-Script Amp Cloning Kit from Stratagene (La Jolla, CA, USA) the protocols used were the ones provided with the kit with minor exceptions. The purified PCR products were polished with cloned *Pfu* DNA polymerase according to the protocol in PCR tubes. The ligation of the amplicon to the pPCR-Script Amp SK(+) cloning vector was carried out with 2µL of the polished and purified PCR product, T4 DNA ligase, which was provided with the kit, was used for the ligation process according to the protocol. The plasmids were then inserted into the XL10-Gold Kan ultracompetent cells according to the

protocol except that, SOC broth was used instead of the NZY^{+} broth mentioned in the protocol. The cells were then plated onto Amp/X-Gal/IPTG LB Agar plates previously prepared in the lab.

After overnight incubation at 37^{o}C, blue, semi-blue and white colonies were obtained. A selection of individual white colonies were collected with the tips of 10µL pipette tips and placed in LB Broth containing ampicillin and incubated overnight at 37^{o}C. A small volume (10µL) of the broth was used the following morning, after culture growth, diluted 5x and boiled for 5' and spundown. This bacterial lysate was then used in a PCR reaction to confirm the correct insert was present before proceeding with plasmid purification. A purified plasmid suspension was obtained with a Wizard plus sv minipreps DNA purification system from Promega (Madison, WI, USA) and following the centrifugation protocol provided in the kit. A PCR using 1µL of the purified plasmid suspension and primers for the forward and reverse M13 sites of the plasmid along with plasmid digestion of 1µL of the purified plasmid suspension with EcoRI restriction enzyme (Sigma-Aldrich, St. Louis, MO, USA) confirmed the presence of the correctly sized insert. The clones were then sent for sequencing by STABvida.

All PCRs of the 3' RACE cDNA were conducted in the same way as the ones described above with the exception of allowing for an elongation period of 1 minute 15 seconds, based on the predicted size of the amplicon. All steps of the cloning process were carried out in a laminar flowhood to avoid contamination.

Preparation of LB Agar plates and LB broth

LB Agar plates were prepared with LB Agar from Sigma-Aldrich (St. Louis, MO, USA) the agar was suspended and autoclaved. The agar was then cooled to 60^{o}C and ampicillin was then added to a final concentration of 100µg/mL. Twenty mL of the media was then poured into each Petri dish, cooled for 15 minutes with the lid on and then for 3 hours with the lid off to avoid condensation build up in the Petri dishes. For each Petri dish a solution of 100µL of SOC broth, 10µL of 0.1M IPTG and 20µL X-Gal [50mg/mL) was

poured over each plate and spread with a flamed glass spreader and allowed to sit and absorb the IPTG and X-Gal for 30 minutes.

A stock solution of LB broth was also prepared using LB Broth from Sigma-Aldrich. The solution was then autoclaved and kept in the laminar flowhood until needed. Ampicillin was added to every volume of broth taken to a final concentration of 100µg/mL.

After autoclaving all steps involving the medias were performed in the laminar flowhood in order to avoid contamination of the Petri dishes and stock LB broth.

Gene sequence analysis

The sequencing carried out during for the 3' RACE returned the nucleotide sequence for trypsinogen (sequence in appendix) as such the previously obtained sequence for Gase, which had been used for designing loach specific primers, was utilized to carry out phylogenic analysis for the weatherloach Gase gene sequence. Also, the same analysis would be carried out for GDH and GSase, using the sequences obtained previously for weatherloach specific primer design.

The sequences were inputted into the Expasy translate tool (http://www.expasy.ch/ tools/dna.html) in order to determine the correct open reading frame (ORF) for each sequence, which was determined by using blastp software on the Expasy webpage (http://www.expasy.ch/tools/blast/), the frame that returned results for the correct gene and one or no STOP codons was considered the correct ORF. For Gase the key amino acid sequences chosen for comparative analysis were zebrafish (*Danio rerio*) (Swiss-Prot id Q8JFS4), pufferfish (*Tetraodon nigroviridis*) (Swiss-Prot id Q4RMW1), chicken (*Gallus gallus*) (Swiss-Prot id Q5ZIV6), the kidney and liver isoforms for rat (*Rattus norvegicus*) (Swiss-Prot id P13264 and P28492) and human (*Homo sapiens sapiens*) (Swiss-Prot id O94925 and Q9UI32). For GDH the key sequences were zebrafish (Swiss-Prot id Q6NZ29), rainbow trout (*Oncorhynchus mykiss*) (Swiss-Prot id Q8JHY1), Atlantic salmon (*Salmo salar*) (Swiss-Prot id C0PUN2), African clawed frog (*Xenopus laevis*) (Swiss-Prot id Q6AZJ0), mouse (*Mus musculus*) (Swiss-Prot id P00367) and human (Swiss-Prot id

P00367). For GSase the sequences used were zebrafish (Swiss-Prot id Q7T2P7), pufferfish (Swiss-Prot id Q8JIZ8), rainbow trout (Swiss-Prot id Q8JI31), Atlantic salmon (Swiss-Prot id B5XCB2), African clawed frog (Swiss-Prot id Q6INY0), rat (Swiss-Prot id P09606) and human (Swiss-Prot id P15104).

The sequence alignments were carried out with the online tool ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using the Neighbor Joining (NJ) method, this method does not require that all lineages have diverged by equal amounts, the Kimura correction of distances (this is because, as sequences diverge, more than one substitution will happen at many sites, you only see one difference when you look at the present day sequences, this option has the effect of stretching branch lengths in trees), and the ignore gaps option active to create a phylogenetic tree. In order to confirm the alignment given by ClutalW2, ClustalW (http://align.genome.jp/) was used with the Phylip output format. Alignment was executed with a slow/accurate pairwise alignment all other parameters were the default parameters. Additionally bootstrapping for the NJ trees was calculated using the ClustalX 2.0.11 software (http://www.clustal.org), parameters were 111 as a random number of generated seeds and 1000 as the number of bootstrapping trials, and the resulting trees were used for graphical representation since there were no discernable differences.

Statistics

The data for the tissue distribution and the ammonia exposure are presented as the ratio between mean values for GDH, Gase or GSase by the reference gene actin, which allows for the normalization of the data. For the ammonia exposure data the values were divided by the control group, which also served as time 0, for the corresponding tissue so that all changes would be relative to this group.

Statistical analysis was carried out using the SigmaStat 3.0 software (Systat Software Inc, San Jose, CA, USA). Two way ANOVA tests were carried out in order to determine significant statistical differences ($P<0,05$) between the results, for the tissue distribution experiment the parameters were tissue versus animal and tissue versus gene.

An initial one-way ANOVA for actin indicated no differences between tissues. For the ammonia exposure experiment foregut and hindgut were analyzed separately by one-way ANOVA because actin expression levels were different between tissues. The parameters tested were tissue versus time.

RESULTS

Phylogeny and gene analysis

A 3' RACE was carried out for the 3' sequencing of the Gase gene in weatherloach but was unsuccessful. The sequence obtained did not correspond to Gase but to trypsinogen. Due to time constraints, the sequence and phylogenic analysis were conducted using the partial sequences we initially obtained (see annex A), which served as the basis for designing the weatherloach specific primers. Since I would be using a partial sequence for analysis I could also conduct a similar analysis on all the gene sequences I had, including GDH and GSase.

```
Weatherloach    PKVPFCLQSCVKPLKYAVAVHDHSTEYVHSFIGKEPSGLRFNKLFLDEDDKPHNPMVNAGAIVCTSLIKQGAGNAEKFDHMMNFLKKMAGNEYVGFSNATFQSERESGDR  110
Zebrafish       TKVPFCLQSCVKPLKYAISVHDHGTEYVHRFIGKEPSGLRFNKLFLDEDDKPHNPMVNAGAIVCTSLIKQLAGNAEKFDYVMNFLKKMAGNEYVGFSNATFQSERESGDR  110
Pufferfish      TKVPFCLQSCVKPLKYAIAVHDHGTEYVHRFIGKEPSGLRFNKLFLNEDDKPHNPMVNAGAIVCTSLIKQGASNAEKFDYVMNFMNKLAGNEYVGFSNATFQSERESGDR  110
Rat_Kidney      TKVPFCLQSCVKPLKYAIAVNDLGTEYVHRYVGKEPSGLRFNKLFLNEDDKPHNPMVNAGAIVVTSLIKQGVNNAEKFDYVMQFLNKMAGNEYVGFSNATFQSERESGDR  110
Human_Kidney    TKVPFCLQSCVKPLKYAIAVNDLGTEYVHRYVGKEPSGLRFNKLFLNEDDKPHNPMVNAGAIVVTSLIKQGVNNAEKFDYVMQFLNKMAGNEYVGFSNATFQSERESGDR  110
Chicken         TKVPFCLQSCVKPLKYAIAVNDLGTEYVHRYVGKEPSGLRFNKLFLNEDDKPHNPMVNAGAIVVTSLIKQGANNAEKFDYVMQFMNKMAGNEYVGFSNATFQSERESGDR  110
Rat_Liver       TKIPFCLQSCVKPLTYAISVSTLGTDYVHKFVGKEPSGLRYNKLSLNEEGIPHNPMVNAGAIVVSSLIKMDCNKAEKFDFVLQYLNKMAGNEFMGFSNATFQSEKETGDR  110
Human_Liver     TKIPFCLQSCVKPLTYAISISTLGTDYVHKFVGKEPSGLRYNKLSLNEEGIPHNPMVNAGAIVVSSLIKMDCNKAEKFDFVLQYLNKMAGNEYMGFSNATFQSEKETGDR  110

Weatherloach    NFAIGYYLKEKKCFPDGTDMTAVLDLYFQLCSIEVTCESASVMAATLANGGFCPITGERVLNPEAVRNTLSLMHSCGMYDFSGQFAFHVGLPAKSGVSGGILLVVPNVNG  220
Zebrafish       NFAIGYYLKEKKCFPDGTDMTAVLDLYFQLCSIEVTCESASVMAATLANGGFCPITGERVLSPEAVRDTLSLMHSCGMYDFSGQFAFHVGLPAKSGVSGGILLVVPNVMG  220
Pufferfish      NFAIGYYLKEKKCFPEGTDMTSILDFYFQLCSIEVTCESASVMAATLANGGFCPITGERVLSPEAVRNTLSLMHSCGMYDFSGQFAFHVGLPAKSGVAGGILLVVPNVMG  220
Rat_Kidney      NFAIGYYLKEKKCFPEGTDMVGILDFYFQLCSIEVTCESASVMAATLANGGFCPITGERVLSPEAVRNTLSLMHSCGMYDFSGQFAFHVGLPAKSGVAGGILLVVPNVMG  220
Human_Kidney    NFAIGYYLKEKKCFPEGTDMVGILDFYFQLCSIEVTCESASVMAATLANGGFCPITGERVLSPEAVRNTLSLMHSCGMYDFSGQFAFHVGLPAKSGVAGGILLVVPNVMG  220
Chicken         NFAIGYYLKEKKCFPEGTDMVAILDFYFQLCSIEVTCESASVMAATLANGGFCPITGERVLSPEAVRNTLSLMHSCGMYDFSGQFAFHVGLPAKSGVAGGILLVVPNVMG  220
Rat_Liver       NYAIGYYLKEKKCFPKGVDMMAALDLYFQLCSVEVTCESGSVMAATLANGGICPITGESVLSAEAVRNTLSLMHSCGMYDFSGQFAFHVGLPAKSAVSGAILLVVPNVMG  220
Human_Liver     NYAIGYYLKEKKCFPKGVDMMAALDLYFQLCSVEVTCESGSVMAATLANGGICPITGESVLSAEAVRNTLSLMHSCGMYDFSGQFAFHVGLPAKSAVSGAILLVVPNVMG  220
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Figure 2 – Glutaminase (Gase) 220 partial amino acid sequences for weatherloach and homologs from representative species (Swiss-Prot accession numbers in parenthesis): zebrafish (Q8JFS4), pufferfish (Q4RMW1), chicken (Q5ZIV6), the kidney and liver isoforms for rat (P13264 and P28492) and human(O94925 and Q9UI32). Grey highlighted areas show consensus in all sequences.

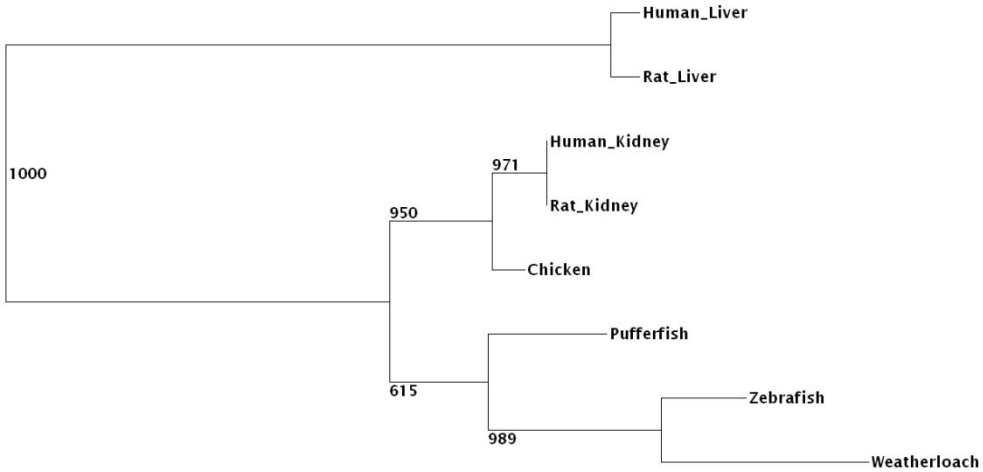

Figure 3 – Phylogenetic tree for glutaminase (Gase) homolog sequences. Displays the phylogeny of the Gase gene between the weatherloach and the key species; zebrafish (Q8JFS4), pufferfish (Q4RMW1), chicken (Q5ZIV6), the kidney and liver isoforms for rat (P13264 and P28492) and human (O94925 and Q9UI32). Values on the branches are the bootstrapping results.

The partial sequence for Gase was 661bp long, the second open reading frame was found to be the correct one. After translation and open reading frame (ORF) determination using the DNA-protein translation tool available on the Expasy website, I had a 220 amino acid long sequence for comparison. The amino acid sequence for weatherloach was considerably shorter than the other key sequences used, these were around 674 amino acids long. However the alignment was conducted none the less and found in a highly conserved region of the cDNA, allowing for continued analysis.

It is well documented that there are two existing isoforms for Gase in mammals, kidney and liver, and so among the representative species were chosen two sequences for the mammalian liver isoform (human and rat) were included. The key species chosen were therefore zebrafish (*Danio rerio*), pufferfish (*Tetraodon nigroviridis*), chicken (*Gallus gallus*), and both human (*Homo sapiens sapiens*) and rat (*Rattus norvegicus*) kidney and liver isoforms. Predictions using the blastp software showed that the weatherloach sequence was similar to the kidney isoform but for phylogenic analysis the liver isoform was included.

Results showed a high level of similarity, 95-89% similarity, between the sequences for fish, chicken and mammalian kidney isoforms (see figure 2). The similarity among the weatherloach sequence and mammalian liver isoforms was found to be ≈77%, in the partial sequence used for the comparison. A phylogenic tree was also constructed using the Neighbor joining method which showed elevated bootstrapping values of the branches (>700) which translates as significant differences in the branching.

Weatherloach and zebrafish show the highest similarity and phylogenic proximity followed by pufferfish. The chicken and kidney isoforms show the next closest relationship with weatherloach and the other fish sequences. Liver as mentioned shows the greatest distinction and separation from the other sequences (see figure 3).

Since I would not be analyzing the whole sequence for the Gase gene a similar analysis was conducted for the other two genes by using the initial partial sequences, 933bp long sequence for GDH and 498bp sequence for GSase, used for weatherloach specific primer design.

Figure 4 – Amino acid consensus sequences for glutamate dehydrogenase (GDH). The sequences used were 306 amino acids long, for weatherloach and the key species; zebrafish (Q6NZ29), rainbow trout (Q8JHY1), Atlantic salmon (C0PUN2), African clawed frog (Q6AZJ0), mouse (P00367) and human(P00367).

Not unlike Gase there are other isoforms of GDH; however, for the sequence analysis of GDH only the type 1 isoform was considered, all the blastp results were for GDH type 1 and so, in this case, only this isoform was used for analysis. For the GDH sequence the homologs from zebrafish, rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), African clawed frog (*Xenopus laevis*), human and mouse (*Mus musculus*) were used (see figure 4). The results showed a 93-89% sequence similarity with the other sequences used in the relatively long sequence I had for comparison. A phylogeny tree, like the one for Gase, obtained from the online tool ClustalW2 showed close similarity between the weatherloach and zebrafish sequences, a very close similarity between rainbow trout and Atlantic salmon and a common ancestry for mammalian and the amphibian homologs. As with Gase a phylogeny tree based solely on the full amino acid sequences for the key species chosen was made and visually compared, the resulting tree and the one produced using the shorter sequences were sufficiently similar and was accepted as plausible. In accordance with the previous statement the size of the weatherloach sequence and high homology among the sequences makes the analysis of this gene the most reliable and possibly accurate. Additionally the bootstrapping results show significant branching of the weatherloach and zebrafish from Atlantic salmon and rainbow trout and, similarly, of the fish species from the others (see figure 5). Also of

worthy of note is the branching of the African clawed frog from the mammalian species, though these 3 are more closely related to each other than to the fish sequences.

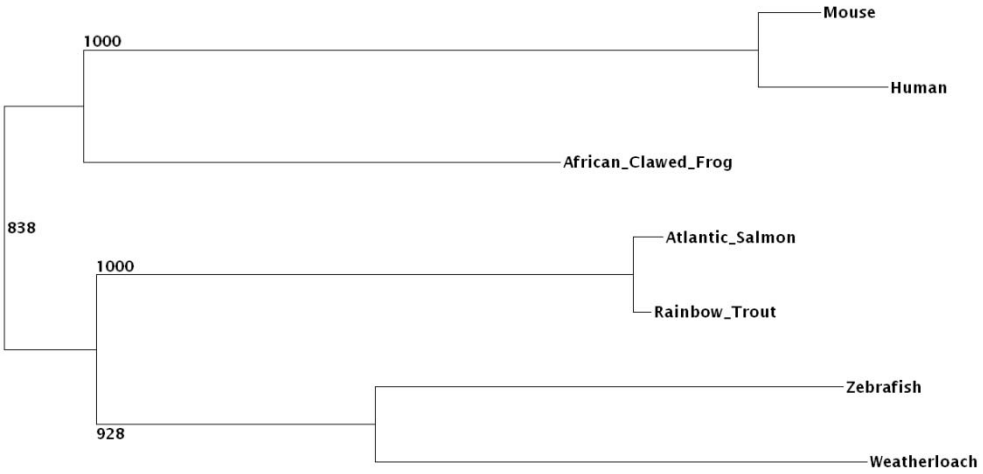

Figure 5 – Phylogram tree for glutamate dehydrogenase (GDH). Displays the phylogeny of the GDH gene between the weatherloach and the key species; zebrafish (Q6NZ29), rainbow trout (Q8JHY1), Atlantic salmon (C0PUN2), African clawed frog (Q6AZJ0), mouse (P00367) and human(P00367). Values on the branches are the bootstrapping results.

GSase also showed a high degree of similarity between the sequences, in this case the homologs used were from rainbow trout, Atlantic salmon, zebrafish, pufferfish, African clawed frog, human and rat. Once again the similarity between the sequences was high, 83-90% among them (see figure 6). The problem with this sequence was the relative low quality of the sequencing that did not allow for a larger gene sequence for analysis. The partial sequence used was rather short 165 amino acid long sequence (498bp) however the full length key sequences available had only 371 amino acids. With the exception of salmon and trout which had a 99% identity among them, there was an 83-90% homology among the other sequences and weatherloach, and though not as high as with the other two genes, it is still quite high.

The resulting phylogenic tree showed that rainbow trout and Atlantic salmon for this small sequence had distinct branching from the remaining fish species. Similarly the fish species showed a significant branching from mammals and amphibians, which were also significantly different from the other sequences (see figure 7).

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Weatherloach     PFRKDPNKLVLCEVLKYNRKPAETNHRKTCNKVMEKVRDQVPWFGMEQEYXLLGTDGHPFGWPSNGXPGPQGPYYCGVGADKAYGRDIVEAHYRACLYAGVQICGTNAEV 110
Rainbow Trout    PFRKDPNKLVLCEVLKYNRKPAETNLRLTCNKVMDMVENQVPWFGMEQEYTILGTDGHPFGWPNNGFPGPQGPYYCGVGSDKAYGRDIVEAHYKACLYAGVMICGTNAEV 110
Atlantic Salmon  PFRKDPNKLVLCEVLKYNRKPAETNLRLTCNKVMDMVENQVPWFGMEQEYTILGTDGHPFGWPNNGFPGPQGPYYCGVGSDKAYGRDIVEAHYRACLYAGVMICGTNAEV 110
Pufferfish       PFRKDPNKLVLCEVLKYNRKPTETNLRLTCKKVMDMVADQHPWFGMEQEYTILGTDGHPFGWPSNGFPGPQGPYYCGVGADKAYGRDVVEAHYKACLYAGVQICGTNAEV 110
Zebrafish        PFRKDPNKLVLCEVVKYNRKTAETNHRHTCKKIMEMVGHQSPWFGMEQEYTILGTDGHPFGWPSNGFPGPQGPYYCGVGADKAYGRDIVEAHYRACLYAGVMICGTNAEV 110
Human            PFRKDPNKLVLCEVFKYNRRPAETNLRHTCKRIMDMVSNQHPWFGMEQEYTLMGTDGHPFGWPSNGFPGPQGPYYCGVGADRAYGRDIVEAHYRACLYAGVKIAGTNAEV 110
Rat              PFRRDPNKLVFCEVFKYNRKPAETNLRHSCKRIMDMVSSQHPWFGMEQEYTLMGTDGHPFGWPSNGFPGPQGPYYCGVGADKAYGRDIVEAHYRACLYAGIKITGTNAEV 110
African frog     PFRRDPNKLVLCEVLKYNRKTAETNLRHTCNQIMDMMGNEHPWFGMEQEYTLLGMDGHPFGWPSNGFPGPQGPYYCGVGANKAYGRDIVEAHYRACLYAGVKIAGTNAEV 110

Weatherloach     MPAQWEFQVGPCEGISMGDHLWVARFILHRVCEDFGVVASLDPKPITGNWN 161
Rainbow Trout    MPAQWEFQVGPCEGISMGDHLWAARFILHRVCEDFGVVASFDPKPIPGNWN 161
Atlantic Salmon  MPAQWEFQVGPCEGISMGDHLWAARFILHRVCEDFGVVASFDPKPIPGNWN 161
Pufferfish       MPAQWEFQVGPCEGIDMGDHLWVARFILHRVCEDFGVVASFDPKPIPGNWN 161
Zebrafish        MPAQWEFQVGPCEGIDMGDHLWVARFILHRVCEDFGVVASFDPKPIPGNWN 161
Human            MPAQWEFQIGPCEGISMGDHLWVARFILHRVCEDFGVIATFDPKPIPGNWN 161
Rat              MPAQWEFQIGPCEGIRMGDHLWVARFILHRVCEDFGVIATFDPKPIPGNWN 161
African frog     MPAQWEFQIGPCEGIDMGDHLWIARFILHRVCEDFGIIVSFDPKPITGNWN 161
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Figure 6 – Glutamine synthetase (GSase) amino acid sequences. alignment of the 161 amino acids long sequences for weatherloach and the key species; zebrafish (Q7T2P7), pufferfish (Q8JIZ8), rainbow trout (Q8JI31), Atlantic salmon (B5XCB2), African clawed frog (Q6INY0), rat (P09606) and human(P15104).

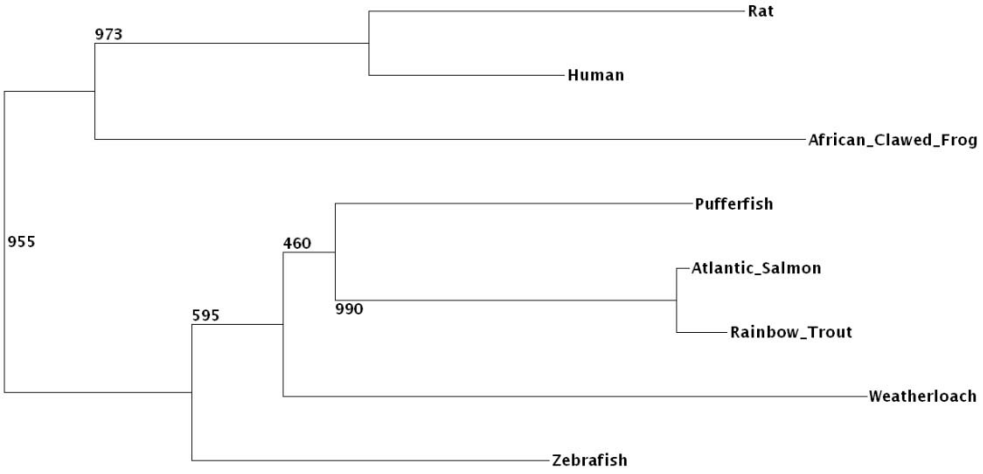

Figure 7 – Phylogram tree for glutamine synthetase (GSase). Displays the phylogeny of the GDH gene between the weatherloach and the key species; zebrafish (Q7T2P7), pufferfish (Q8JIZ8), rainbow trout (Q8JI31), Atlantic salmon (B5XCB2), African clawed frog (Q6INY0), rat (P09606) and human(P15104). Values on the branches are the bootstrapping results.

Tissue distribution

For this part of the work the gene expression of glutaminase, glutamate dehydrogenase and glutamine synthetase across several tissues was quantified in order to determine their baseline expression. This would possibly, allow me to infer about glutamine metabolism and whether it tended more towards anabolism, and the synthesis of glutamine, or catabolism, and the consumption of glutamine based on levels of gene expression, even though direct comparison is not possible. The total number of samples was only 2 however in order to minimize variability among the results each sample was

quantified 4 times, subsequently no statistical differences (P>0,05) were found among the tissue samples.

Actin served as a reference gene and no statistical differences were detected among the tissues (P>0,05) (data in annexes) which made it adequate for normalizing the data for the expression of the other three genes.

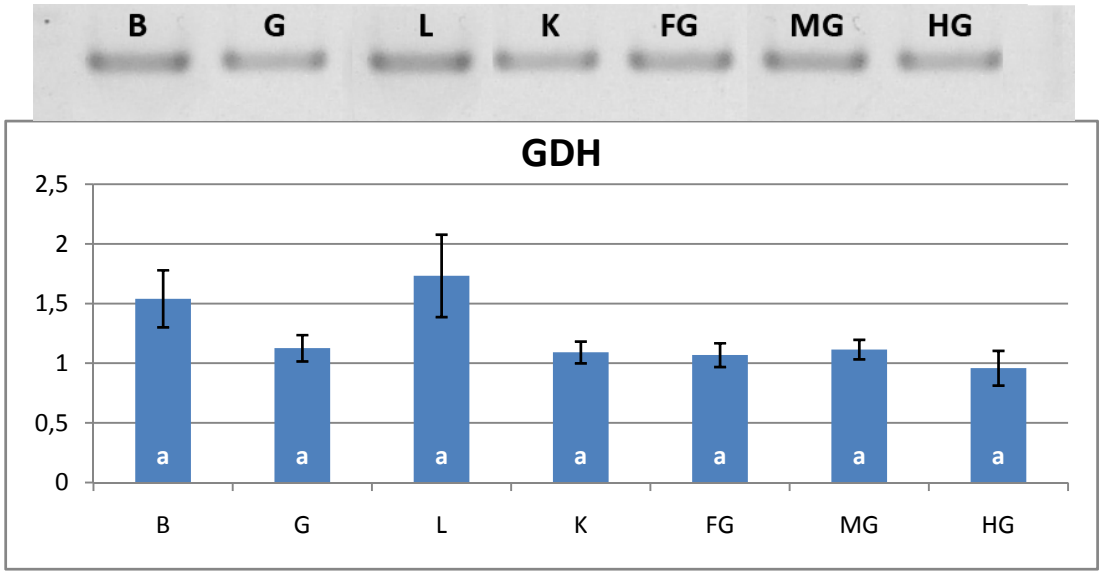

Figure 8– GDH (glutamate dehydrogenase) expression levels per tissue. Values are presented as the ratio between average band intensities for Gase and actin (results not shown) for the different tissues; brain (B), gill (G), liver (L), kidney (K), foregut (FG), midgut (MG) and hindgut (HG). Like characters (shown in white) show no statistically significant differences (P>0,05).

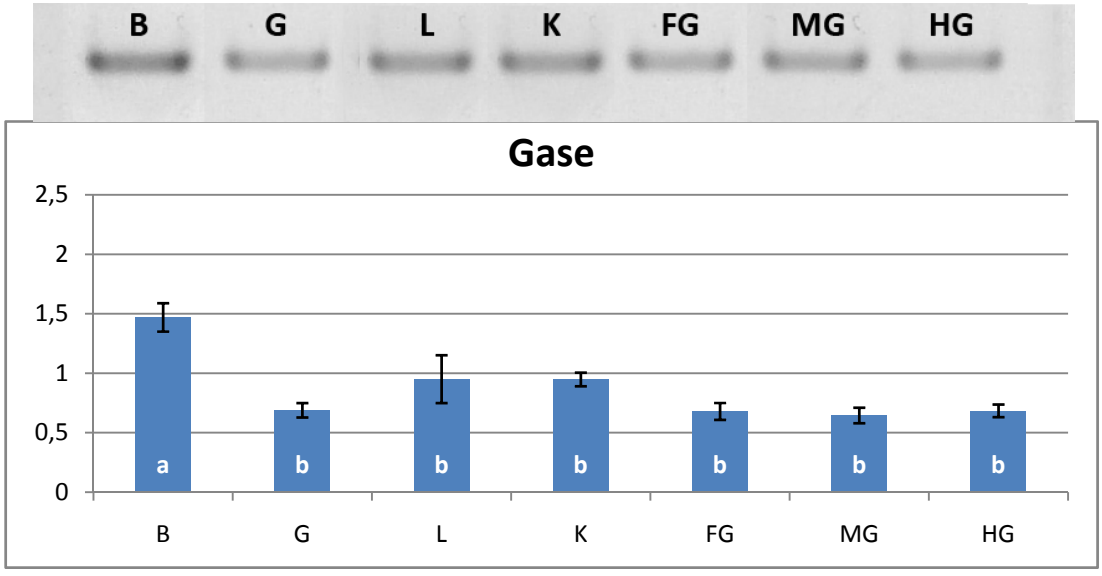

Figure 9 – Gase (glutaminase) expression levels per tissue. Values are presented as the ratio between average band intensities for Gase and actin (results not shown) for the different tissues; brain (B), gill (G), liver (L), kidney (K), foregut (FG), midgut (MG) and hindgut (HG). Like characters (shown in white) show no statistically significant differences (P>0,05).

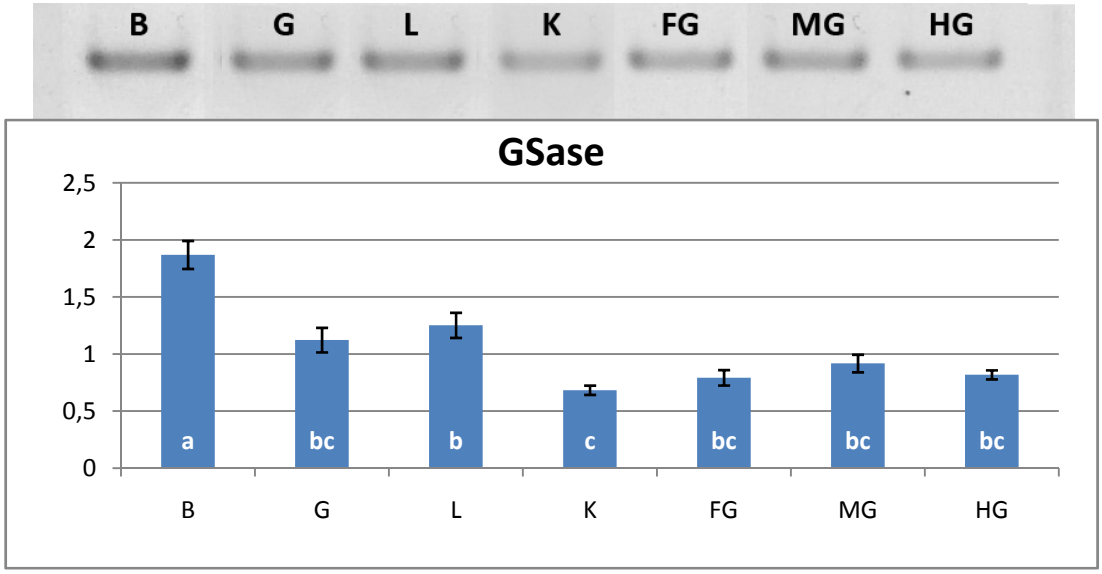

Figure 10 – GSase (glutamine synthetase) expression levels per tissue. Values are presented as the ratio between average band intensities for GSase and actin (results not shown) for the different tissues; brain (B), gill (G), liver (L), kidney (K), foregut (FG), midgut (MG) and hindgut (HG). Like characters (shown in white) show no statistically significant differences (P>0,05).

There seems to be a discernable trend in the data (see figure 8). Higher levels seem to be consistent with brain and liver. As for the other tissues the gene expression levels seem to be the same no matter which tissue is selected. Also worth some reference is that the hindgut portion shows a slightly lower expression level than the other portions of the gut. However as no significant differences where found this may solely be due to the low number of replicas (n=2).

Glutaminase in the brain appeared higher than in the other tissues (see figure 9). However, expression levels in the gut and gill seem to be lower than those of liver and kidney. The glutaminase levels in the liver may be higher since the variation is quite high (±0,2).

The expression levels for GSase showed a slightly higher degree of differentiation among the tissues (see figure 10). The highest level of expression was in the brain compared to all other tissues. Liver levels were more elevated than those of kidney.

Ammonia exposure

Two tissue, foregut and hindgut, which are potential sites of ammonia volatilization, were used in order to determine the potential role of glutaminase and glutamate dehydrogenase in *in situ* ammoniagenesis. Glutamine synthetase transcript levels were also measured in these tissues since this enzyme has been proposed to prevent the backcflux of luminal ammonia (Mommesen, 1984)

Actin was used as a reference gene in order to establish the same common ground as for the tissue distribution experiment. However significant statistical differences were found between actin for foregut and hindgut which does not allow for direct comparison of the expression of the three genes over time (data in annexes). I can therefore only discuss changes in expression of the genes in each tissue separately, and possible changes in expression could be inferred as changes in metabolic activity.

Since no statistical differences were found over time in foregut ($P>0,05$), only possible trends in the data can be mentioned. The expression of glutamate dehydrogenase over time seems to slightly rise over time after an initial dip in expression (figure 12). As for Gase (figure 11) and GSase (figure 13) they both have slightly lower expression over the first 6h and then rise somewhat until the 24h mark. After an extended period of time GSase expression values seem to return to basal conditions while Gase is slightly elevated.

As with foregut, hindgut showed no statistical differences over time and again like with foregut only possible trends in the data can be mentioned. GDH gene expression seems to steadily rise over 24 hours and after an extended period of time stabilize at a slightly higher level of expression than under basal conditions (see figure 15). Gase (figure 14) and GSase (figure 16) seem to have identical expression levels over the first 24 hours. Initially over the first 6 hours expression levels drop and then increase to slightly higher values, than those of the control, after 24 hours. Gene expression seems to return to basal conditions over an extended period of time.

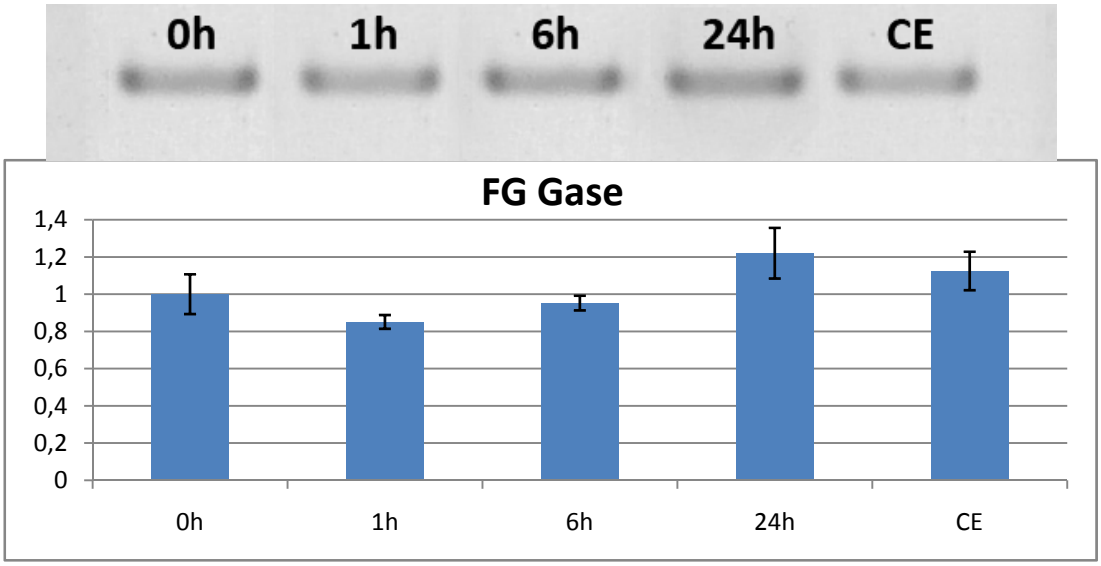

Figure 11 – Gase (glutaminase) gene expression levels over time in foregut (FG). Results are presented as the ratio between average band intensities for GSase and actin (results not shown) divided by the first time point so all changes will be relative to the initial time point. The samples were taken at the beginning of the experiment (0h), 1 hour after the beginning, 6 hours and 24 hours and after a prolonged chronic exposure (CE). No statistically significant differences were found (P>0,05, n=5) over time.

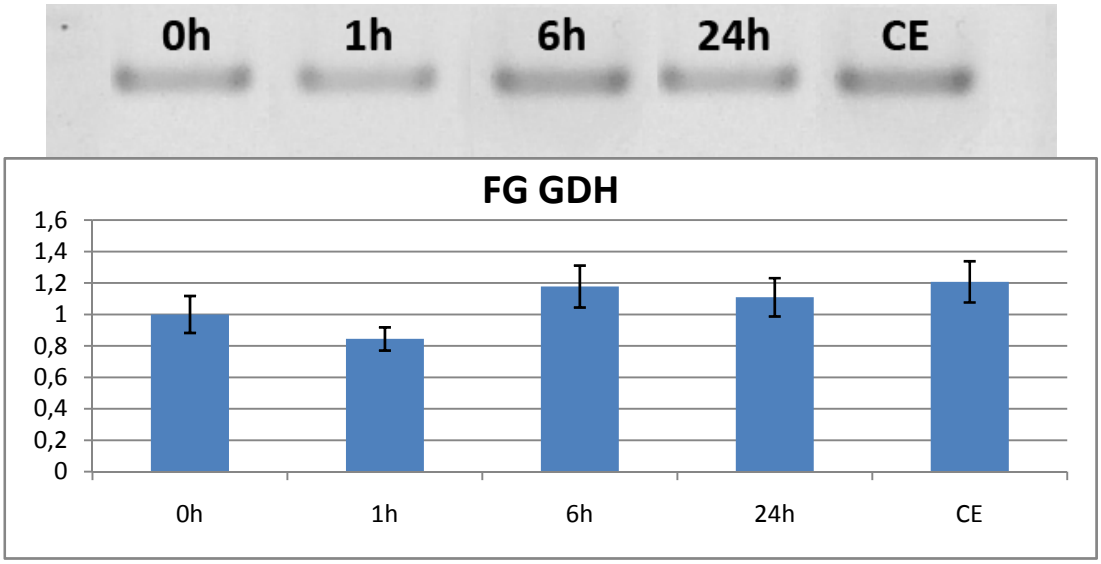

Figure 12 – GDH (glutamate dehydrogenase) gene expression levels over time in foregut (FG). Results are presented as the ratio between average band intensities for GSase and actin (results not shown) divided by the first time point so all changes will be relative to the initial time point. The samples were taken at the beginning of the experiment (0h), 1 hour after the beginning, 6 hours and 24 hours and after a prolonged chronic exposure (CE). No statistically significant differences were found (P>0,05, n=5) over time.

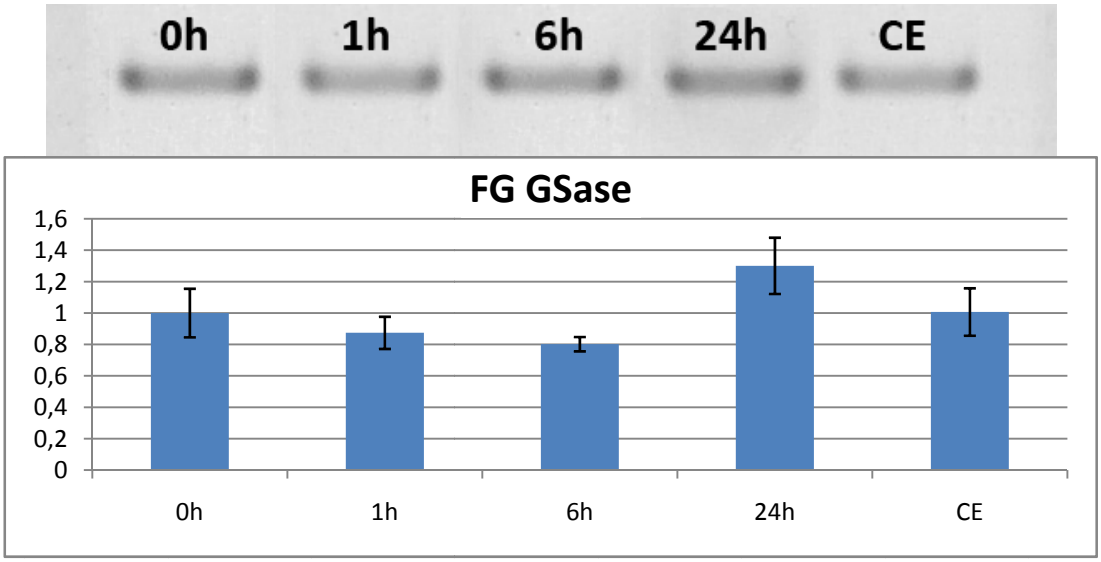

Figure 13 – GSase (glutamine synthetase) gene expression levels over time in foregut (FG). Results are presented as the ratio between average band intensities for GSase and actin (results not shown) divided by the first time point so all changes will be relative to the initial time point. The samples were taken at the beginning of the experiment (0h), 1 hour after the beginning, 6 hours and 24 hours and after a prolonged chronic exposure (CE). No statistically significant differences were found (P>0,05, n=5) over time.

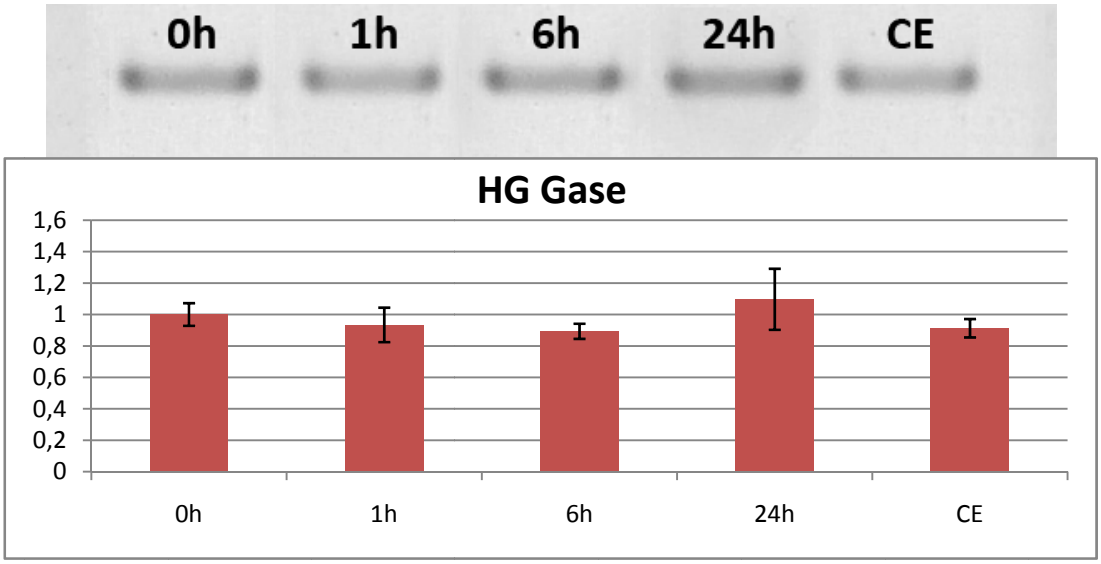

Figure 14 – Gase (glutaminase) gene expression levels over time in hindgut (HG). Results are presented as the ratio between average band intensities for GSase and actin (results not shown) divided by the first time point so all changes will be relative to the initial time point. The samples were taken at the beginning of the experiment (0h), 1 hour after the beginning, 6 hours and 24 hours and after a prolonged chronic exposure (CE). No statistically significant differences were found (P>0,05, n=5) over time.

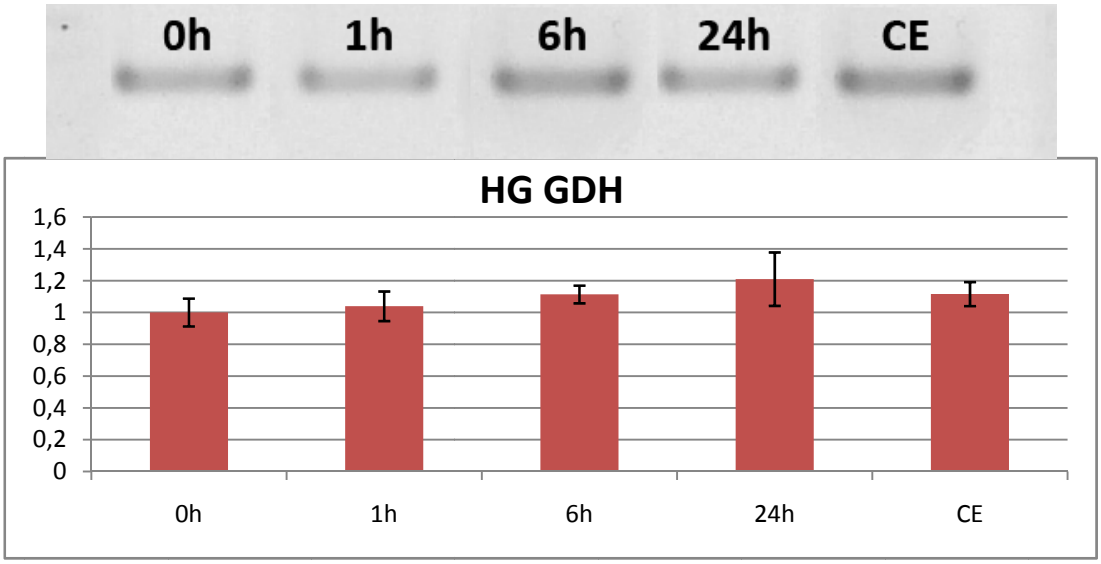

Figure 15 – GDH (glutamate dehydrogenase) gene expression levels over time in hindgut (HG). Results are presented as the ratio between average band intensities for GSase and actin (results not shown) divided by the first time point so all changes will be relative to the initial time point. The samples were taken at the beginning of the experiment (0h), 1 hour after the beginning, 6 hours and 24 hours and after a prolonged chronic exposure (CE). No statistically significant differences were found (P>0,05, n=5) over time.

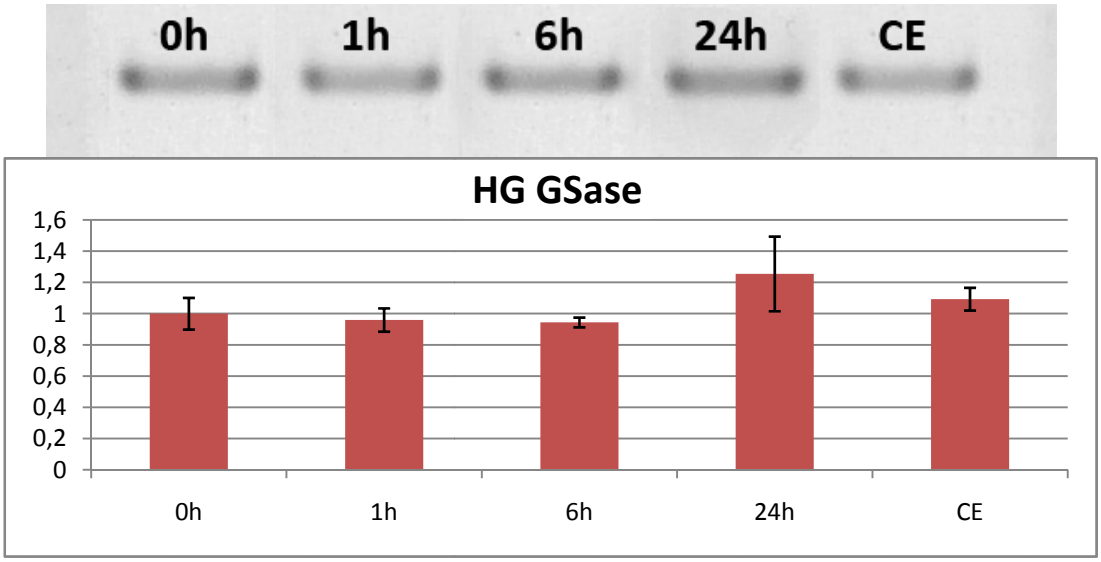

Figure 16 – GSase (glutamine synthetase) gene expression levels over time in hindgut (HG). Results are presented as the ratio between average band intensities for GSase and actin (results not shown) divided by the first time point so all changes will be relative to the initial time point. The samples were taken at the beginning of the experiment (0h), 1 hour after the beginning, 6 hours and 24 hours and after a prolonged chronic exposure (CE). No statistically significant differences were found (P>0,05, n=5) over time.

DISCUSSION

The main focus of this work was initially to try and shed some light on the possible involvement of glutamine metabolism in the foregut and hindgut of weatherloach, which are the predicted sites of ammonia volatilization. Glutamine maybe involved through ammoniagenesis via glutaminase (Gase) and preventing ammonia backflux through glutamine synthetase (GSase). However there were no significant changes in gene expression in response to ammonia loading, which is known to increase ammonia volatilization, to support a role for glutamine so the work was refocused on a more general gene characterization. Never the less there seem to be possible tendencies in gene expression which will be discussed.

Gase, GDH and GSase were the three genes this work would focus on, and so sequence and phylogenic comparison was carried out on the sequences that had served as the basis for loach specific primer design, theses primers later served for the expression analysis in the other experiments. Results showed high homology among the sequences and the key species chosen for each gene, additionally the phylogenic trees showed that although there were high levels of homology amongst the genes there was also significant branching evolution wise. The tissue distribution showed some significant gene expression levels mostly limited to the brain, although basal levels were found in most other tissues studied.

Gene analysis

Phylogenic analysis through sequence alignment and phylogenic trees based on such short sequences, as the ones I had, are not completely accurate and therefore not recommended. However, since the region the sequence was in was highly conserved and had no gaps even among both isoforms the tree and alignments were inferred using the Neighbor Joining method. For comparative purposes and in order to infer about the accuracy of the phylogeny tree, a tree based solely on the full amino acid sequences for the selected key species was made and visually compared, if the resulting tree and the

one produced using the shorter sequences were sufficiently similar the tree could be accepted as plausible. In addition the bootstrapping values were calculated in order to determine significant branching of the phylogenic trees.

The Gase amino acid sequence shows high sequence homology (95%). As expected, the weatherloach sequence was most similar to zebrafish, both species are of the cypriniformes order and so have a closer common ancestry. Pufferfish, being of the actinopterygii class has a slighty lower homology and accordingly a farther ancestry. As mentioned the liver isoform had the lowest homology with the other sequences and, consistently, the farthest ancestry. These results are supported with the phylogenic tree bootstrapping values which show significant branching of the tree consistent with what would be expected, species with closer ancestries are more closely associated with each other, even though significant branching of the trees shows that the sequences did suffer changes with speciation the changes seem to be consistent with the evolutionary path.

There is high sequence similarity among all the sequences for the GDH type 1 isoform among the key species, given the size of the weatherloach sequence this may be the most reliable of the sequences analysed. The phylogenic tree showed similar, expected, results consistent with Gase. The results for sequence alignment and the phylogenic trees are similar to what was observed with Gase elevated sequence similarity correlates with significant branching in the phylogenic tree and therefore distinct evolutionary paths. The superior species, evolution wise, show higher similarity amongst each other than with fish, and as would be expected the African clawed frog shows significant identity from the other species. The sequences for weatherloach and zebrafish again showed an elevated homology with each other, and also of note is the high homology between the two closest related species, rainbow trout and Atlantic salmon, these two are of the salmonidae family and therefore much closer related to each other than to zebrafish and weatherloach.

The same analysis was carried out for GSase, though for this gene there seem to be only the single isoform. While the trees for the Gase and GDH using the shortened sequences had similar branches with the trees using the full key sequences this was not the case with GSase.

I could not therefore acurately comment on the phylogeny of the GSase sequence for weatherloach, however the bootstrapping results showed no significant branching among the fish species, with the exception being between the closest related species, rainbow trout and Atlantic salmon. Additionally the sequence for the African clawed frog showed distinct ancestry from the other species.

Tissue distribution

As mentioned actin was used as a reference gene in order to establish a baseline from which all the gene data could be normalized. It was found to be an adequate reference gene for this experiment because, no significant differences were found in its expression between the various tissues. The gene expression levels of Gase, GDH and GSase could then be looked into and analyzed in order to attempt and determine or infer about possible metabolic tendencies. The only drawback is that after some research one tissue in particular may have been of some relevance, muscle tissue has been described as possibly being important in the glutamine metabolism and most importantly involved in ammonia tolerance through the production or degradation of glutamine (Chamberlin *et al*, 1991; Jow *et al*, 1999; Saha *et al*, 2002). Muscle tissue could not be used because during the sampling of the various tissues, no muscle samples were taken for the two most complete sets of tissues.

Glutamate dehydrogenase is involved in both the anabolic and catabolic processes of glutamine catalyzing the conversion of glutamate to α-ketoglutarate and vice versa. Therefore, I expected to find elevated expression levels of this gene anywhere glutamate was being utilized, be it for glutamine synthesis and subsequent ammonia removal (Ip *et al*, 2001). Results showed no statistically significant elevation of gene expression in any particular tissue; however, levels in brain and liver seemed slightly elevated, otherwise the expression levels were basically the same through the other tissues. Glutamate serves as a neurotransmitter and therefore is essential to normal brain function. GDH being involved in both the catabolic and anabolic processes of the glutamine metabolism may serve a purpose in maintain glutamate levels at a certain concentration.

Glutaminase expression levels were found to be higher in brain tissue. The pathway in which glutaminase is involved actively creates glutamate from glutamine; however, it also produces NH_4^+ which during ammonia exposure could cause problems. The weatherloaches in this experiment were not subject to environmental ammonia therefore ammonia loading in the brain should not be an issue. Glutamate serves as a neurotransmitter and so the reaction can therefore be explained by the necessity of synthesizing glutamate and in so doing allow for continued signal propagation. As for the other tissues, liver and kidney also had slightly elevated expression levels of Gase while the other tissues had somewhat low levels of expression. In kidney, Gase has been shown to be important in ammoniagenesis for dealing with chronic metabolic acidosis (Wood *et al*, 1999).

Glutamine synthetase adds new information to the previous results for brain tissue. The expression levels in the brain were also significantly elevated when compared to the other tissues; which makes sense if the brain uses glutamine to synthesize glutamate to serve as a neurotransmitter it must also actively remove it from the synaptic gap. This glutamine synthesis serves a dual purpose, it removes glutamate from the synaptic gap and in so doing also converts ammonia to a less toxic form, glutamine (Randall and Tsui, 2002; Veauvy *et al*, 2005). As mentioned previously, the catabolic process releases NH_4^+ which affects membrane potential and can therefore influence the capability of the neuron to transmit a signal. One of the documented ways to remove NH_4^+ in the brain is precisely the synthesis of glutamine (Randall and Tsui, 2002; Veauvy *et al*, 2005). GSase levels in gill, foregut, midgut and hindgut showed no significant differences among them or with kidney and liver; however, kidney and liver where significantly different from each other. This may mean that kidney and liver are opposites when it comes to GSase expression, one being elevated (liver) and one having a low expression level (kidney), the use of only two sets of tissues could be influencing the results, a higher number of samples would allow for more replicas and a clearer analysis.

The relatively higher levels of glutamine synthetase transcript in the liver is consistent with the finding of higher GS activity in this organ (Jow *et al*, 1999; Chew *et al*, 2001) and its proposed functional significance in synthesizing glutamine in the liver as a

means of removing excess ammonia from circulation and storage in a less toxic form that is also known for being used as an energy source (Chamberlin *et al*, 1991)). Though some fish do use the ornithine urea cycle (OUC) for urea synthesis this is not the case in the weatherloach where the OUC is incomplete and therefore not functional (Chew *et al*, 2001). Therefore, an elevation in GSase will almost certainly be associated glutamine synthesis.

The low levels of GSase in the kidney may be associated with two possibilities either they are low but on par with the levels being expressed on all other tissues or the kidney is not a site where glutamine is synthesized. In mammals, the kidney is known to use glutamine as an energy source and for the excretion of ammonia for acid base regulation during chronic metabolic acidosis (Alleyne *et al*, 1992). Although in fish most ammonia is excreted through the gills a small part is excreted in the urine (Wilkie, 2002); therefore, if fish also use glutamine as an energy source for the kidney it makes little sense to also have elevated levels of GSase since Gase levels are high which would result in futile cycling. In Wood *et al* (1999) acid-base regulation of the kidney is looked into, in some fish glutamine catabolysis is employed as a means of releasing ammonia that can be excreted in the urine and in the same study could not detect any activity for GSase in the kidney. Also, in rat it has been shown by Duong *et al* (1984) that GSase has no role in treating metabolic acidosis in the kidney. This in turn means that kidney expression levels may be in actuality lower than in other tissues which would agree with the activity measurements mentioned above.

Ammonia exposure

This experiment was intended to shed a light on the possibility of glutamine and its metabolism being involved in ammonia volatilization. One of the proposed possibilities was that ammonia could be transported to the hind portion of the gut, where it is known that gaseous exchanges occur or the foregut for active excretion, and through its catabolysis release NH_4^+, in a way similar to the described for the kidney in acid-base regulation (Wood *et al*, 1999), that can in turn be volatilized. Another possibility is that

glutamine is synthesized and stored in the gut as an energy source and means of ammonia removal during backflux from the gut lumen (Mommsen *et al*, 2003). Most works that suggest this possibility usually place this occurrence in the brain or muscle tissue though due to the particular nature of the weatherloach gut involvement in gaseous exchange, and the importance of glutamine as a metabolic fuel in entrocytes it is possible that it occurs in the gut as well. As said this part of the work was intended to attempt and shed a light on glutamine metabolism in the gut and its possible influence in ammonia tolerance and volatilization.

Actin was also used as a housekeeping gene for the ammonia exposure experiment; however, here there were statistical differences, in this case, between foregut and hindgut. The statistical differences in this experiment made it impossible to compare foregut and hindgut directly; a more suitable reference gene should have been chosen and analyzed however, when the statistical analysis was carried time constraints did not allow for this process. As such it was not possible to compare both tissue samples in each time point. Additionally no significant statistical differences were found among the time course samples. With no significant changes in either Gase or GSase transcript expression we find no support for the initial hypotheses. This may indicate that changes are not taking place at the transcriptional level or that glutamine does not play an important role in ammonia volatilization.

CONCLUSION

The phylogenic results showed high homology of the three genes with their orthologues in other species and the resulting phylogenic trees showed results consistent with what would be expected. Weatherloach was consistently found to be more closely related to zebrafish, which was the closest species among the selected sequences. Also the fish species were consistently grouped as having a common ancestry, again as would be expected. The sequences used were roughly half the length of the complete gene sequence and so it must be stated that by obtaining a full length sequence a more accurate analysis could be held, a complete sequence would allow for a more precise tree design and sequence alignments.

The results for the tissue distribution showed higher gene expression for all the glutamine metabolic enzymes in the brain. The activity of this metabolic pathway is well documented in the literature and of significant relevance in the brain. Though not much more could be said about the levels of expression in the other tissues, it was possible to mention a possible tendency of the liver towards glutamine synthesis, and of kidney toward glutamine catabolysis possibly for ammoniagenesis. Though the data was somewhat inconclusive a larger number of samples could have allowed for more firm conclusions about gene expression.

The results for ammonia exposure were somewhat harder to ascertain since there were no statistical differences among the results. However a slight tendency was observed, the foregut seems to tend toward a higher catabolysis during prolonged exposure to ammonia, which may be related to ammonia volatilization. The hindgut on the other hand shows diametrically opposite results tending toward glutamine synthesis suggestive of a role in preventing ammonia backflux. These results not unlike the ones for tissue distribution could benefit from a larger number of replicas and a quantitative approach to transcript measurement (real time PCR).

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ANNEX A – Gene and Amino Acid sequences and Sequence alignment results

Weatherloach sequences for Gase:

>Gase Weatheloach (gene sequence)
GCCTAAAGTGCCGTTTTGCCTGCAGTCGTGCGTGAAGCCACTCAAGTACGCTGTTGCTGTGCACG
ATCACAGCACCGAATATGTGCACAGCTTCATTGGGAAAGAACCCAGCGGCTTACGCTTCAACAAA
CTTTTTCTGGATGAGGATGATAAACCACATAATCCAATGGTGAATGCTGGTGCTATCGTGTGTACA
TCTCTTATTAAGCAGGGGGCAGGCAATGCTGAGAAGTTTGATCATATGATGAACTTCCTAAAGAA
GATGGCAGGAAATGAATATGTCGGCTTCAGTAATGCAACATTCCAATCGGAGCGTGAGTCGGGA
GATAGGAATTTCGCTATTGGTTACTATCTGAAAGAGAAGAAGTGCTTTCCAGATGGTACGGATAT
GACCGCTGTCCTGGACCTCTACTTTCAGCTGTGCTCTATTGAAGTAACATGTGAGAGCGCTAGTGT
CATGGCTGCCACGCTAGCCAATGGCGGATTCTGTCCAATCACAGGCGAGCGTGTGCTGAACCCAG
AGGCCGTGCGAAACACCCTTAGTCTCATGCACTCCTGCGGCATGTACGACTTCTCCGGACAGTTC
GCCTTCCATGTGGGTCTTCCAGCCAAGTCGGGTGTTTCTGGTGGTATTTTGCTTGTTGTGCCTAAT
GTGAATGGC

> Gase Weatheloach (amino acid sequence)
PKVPFCLQSCVKPLKYAVAVHDHSTEYVHSFIGKEPSGLRFNKLFLDEDDKPHNPMVNAGAIVCTSLIK
QGAGNAEKFDHMMNFLKKMAGNEYVGFSNATFQSERESGDRNFAIGYYLKEKKCFPDGTDMTAVL
DLYFQLCSIEVTCESASVMAATLANGGFCPITGERVLNPEAVRNTLSLMHSCGMYDFSGQFAFHVGLP
AKSGVSGGILLVVPNVNG

Key Species full amino acid sequences:

>Zebrafish (Q8JFS4)
CVQSNIVLLTQAFRKKFVIPDFQSFASHIDTLYEKGRNLSGGLVADYIPQLAKFSPDLWAVSLCTVDGQ
RHTVGDTKVPFCLQSCVKPLKYAISVHDHGTEYVHRFIGKEPSGLRFNKLFLDEDDKPHNPMVNAGAI
VCTSLIKQLAGNAEKFDYVMNFLKKMAGNEYVGFSNATFQSERESGDRNFAIGYYLKEKKCFPDGTD
MTAVLDLYFQLCSIEVTCESASVMAATLANGGFCPITGERVLSPEAVRDTLSLMHSCGMYDFSGQFAF
HVGLPAKSGVSGGILLVVPNVMGIMCWSPPLDKLGNSVRGIQFCTDLVSLFNFHNYDNLRHFAKKLD
PRREGGDQRQHTFGPMDYESLQKELALKDTMWTKVSPTECNEDSSTTVVYRMETVKSVINLLFAAYT
GDVSALRRFALSSVDMEQRDYDSRTALHVAAAEGHIEVVRFLLEACKVNPAPKDSISVPQVGQHTNG

>Pufferfish (Q4RMW1)
KAGILPSLEDLLFYTIAEGQEKIPAHKFTTALKSTGLRTGDPRLKECMEMLKVTLKTTSDGALDRHLFKKC
VQSNIVLLTQAFRKKFVIPDFQSFCAHIDDLYENAKNLSGGQVADYIPQLARFSPELWGVALCTVDGQ
RHTVGDTKVPFCLQSCVKPLKYAIAVHDHGTEYVHRFIGKEPSGLRFNKLFLNEDDKPHNPMVNAGAI
VCTSLIKQGASNAEKFDYVMNFMNKLAGNEYVGFSNATFQSERESGDRNFAIGYYLKEKKCFPEGTD
MTSILDFYFQLCSIEVTCESASVMAATLANGGFCPITGERVLSPEAVRNTLSLMHSCGMYDFSGQFAF
HVGLPAKSGVAGGILLVVPNVMGIMCWSPPLDKLGNSVRGIQFCTDLVSLFNFHNYDNLRHFAKKLD
PRREGGDQRVKSVINLLFAAYTGDVSALRRFALSSMDMEQRDYDSRTALHVAAAEGHAEVVRFLLEA
CKVNPVPKDRWGNTPMDEAVHFGHHDVVTILQDYHNKYSPQDPATDKQNAEKNLDGML

>Rat_Kidney (P13264)
MMRLRGSAMLRELLLRPPAAVGGVLRRTQPLGTLCRRPRGGSRPAAGLVAAARLHPWWGGGGRA
KGPGSGGLSSSPSEILQELGKGGTPPQQQQQQQQQPGASPPAAPGPKDSPGETDAFGNSEGKEMV
AAGDNKVKQGLLPSLEDLLFYTIAEGQEKIPVHKFITALKSTGLRTSDPRLKECMDMLRLTLQTTSDGV
MLDKDLFKKCVQSNIVLLTQAFRRKFVIPDFMSFTSHIDELYESAKKQSGGKVADYIPQLAKFSPDLWG
VSVCTVDGQRHSIGDTKVPFCLQSCVKPLKYAIAVNDLGTEYVHRYVGKEPSGLRFNKLFLNEDDKPH
NPMVNAGAIVVTSLIKQGVNNAEKFDYVMQFLNKMAGNEYVGFSNATFQSERESGDRNFAIGYYLK
EKKCFPEGTDMVGILDFYFQLCSIEVTCESASVMAATLANGGFCPITGERVLSPEAVRNTLSLMHSCG
MYDFSGQFAFHVGLPAKSGVAGGILLVVPNVMGMMCWSPPLDKMGNSVKGIHFCHDLVSLCNFH
NYDNLRHFAKKLDPRREGGDQRVKSVINLLFAAYTGDVSALRRFALSAMDMEQRDYDSRTALHVAA
AEGHVEVVKFLLEACKVNPFPKDRWNNTPMDEALHFGHHDVFKILQEYQVQYTPQGDSDDGKENQ
TVHKNLDGLL

>Rat_Liver (P28492)
SMRALQNALSRAGSHGQRGGWGHPSRGPLLGGGVRYYFGEAAAQGRGTPHSHQPQHSDHDASN
SGMLPRLGDLLFYTIAEGQERIPIHKFTTALKATGLQTSDPRLQDCMSKMQRMVQESSSGGLLDRELF
QKCVSSNIVLLTQAFRKKFVIPDFEEFTGHVDRIFEDAKELTGGKVAAYIPHLAKSNPDLWGVSLCTVD
GQRHSVGHTKIPFCLQSCVKPLTYAISVSTLGTDYVHKFVGKEPSGLRYNKLSLNEEGIPHNPMVNAGA
IVVSSLIKMDCNKAEKFDFVLQYLNKMAGNEFMGFSNATFQSEKETGDRNYAIGYYLKEKKCFPKGVD
MMAALDLYFQLCSVEVTCESGSVMAATLANGGICPITGESVLSAEAVRNTLSLMHSCGMYDFSGQFA
FHVGLPAKSAVSGAILLVVPNVMGMMCLSPPLDKLGNSHRGISFCQKLVSLFNFHNYDNLRHCARKL
DPRREGGEVRNKTVVNLLFAAYSGDVSALRRFALSAVDMEQKDYDSRTALHVAAAEGHIDVVKFLIEA
CKVNPFVKDRWGNIPLDDAVQFNHLEVVKLLQDYHDSYMLSETQAEVAAETLSKENLESMV

>Human_Kidney (O94925)
MMRLRGSGMLRDLLLRSPAGVSATLRRAQPLVTLCRRPRGGGRPAAGPAAAARLHPWWGGGGW
PAEPLARGLSSSPSEILQELGKGSTHPQPGVSPPAAPAAPGPKDGPGETDAFGNSEGKELVASGENKIK
QGLLPSLEDLLFYTIAEGQEKIPVHKFITALKSTGLRTSDPRLKECMDMLRLTLQTTSDGVMLDKDLFKK
CVQSNIVLLTQAFRRKFVIPDFMSFTSHIDELYESAKKQSGGKVADYIPQLAKFSPDLWGVSVCTVDGQ
RHSTGDTKVPFCLQSCVKPLKYAIAVNDLGTEYVHRYVGKEPSGLRFNKLFLNEDDKPHNPMVNAGAI
VVTSLIKQGVNNAEKFDYVMQFLNKMAGNEYVGFSNATFQSERESGDRNFAIGYYLKEKKCFPEGTD
MVGILDFYFQLCSIEVTCESASVMAATLANGGFCPITGERVLSPEAVRNTLSLMHSCGMYDFSGQFAF
HVGLPAKSGVAGGILLVVPNVMGMMCWSPPLDKMGNSVKGIHFCHDLVSLCNFHNYDNLRHFAKK
LDPRREGGDQRVKSVINLLFAAYTGDVSALRRFALSAMDMEQRDYDSRTALHVAAAEGHVEVVKFLL
EACKVNPFPKDRWNNTPMDEALHFGHHDVFKILQEYQVQYTPQGDSDNGKENQTVHKNLDGLL

>Human_Liver (Q9UI32)
MRSMKALQKALSRAGSHCGRGGWGHPSRSPLLGGGVRHHLSEAAAQGRETPHSHQPQHQDHDSS
ESGMLSRLGDLLFYTIAEGQERIPIHKFTTALKATGLQTSDPRLRDCMSEMHRVVQESSSGGLLDRDLF
RKCVSSNIVLLTQAFRKKFVIPDFEEFTGHVDRIFEDVKELTGGKVAAYIPQLAKSNPDLWGVSLCTVD
GQRHSVGHTKIPFCLQSCVKPLTYAISISTLGTDYVHKFVGKEPSGLRYNKLSLNEEGIPHNPMVNAGAI
VVSSLIKMDCNKAEKFDFVLQYLNKMAGNEYMGFSNATFQSEKETGDRNYAIGYYLKEKKCFPKGVD
MMAALDLYFQLCSVEVTCESGSVMAATLANGGICPITGESVLSAEAVRNTLSLMHSCGMYDFSGQFA
FHVGLPAKSAVSGAILLVVPNVMGMMCLSPPLDKLGNSHRGTSFCQKLVSLFNFHNYDNLRHCARKL

DPRREGAEIRNKTVVNLLFAAYSGDVSALRRFALSAMDMEQKDYDSRTALHVAAAEGHIEVVKFLIEA
CKVNPFAKDRWGNIPLDDAVQFNHLEVVKLLQDYQDSYTLSETQAEAAAEALSKENLESMV

>Chicken (Q5ZIV6)
MMRLWRAALLRELLLLGPPGAAGGGGPGSGSRRLPPQLAALGAGPATRRGPGPARGLCTRSEGALE
EPGKGAQQPAAPSEPPDGGRNNNHGSGDKELAGGGGGGENKVKQGLLPSLEDLLFYTIAEGQEKIP
VHKFITALKSTGLRTSDPRLKECMDMLRLTLQTTSDGVMLDKDLFKKCVQSNIVLLTQAFRRKFVIPDF
MSFTSHIDELYESAKKQSGGKVADYIPQLAKFSPDLWGVSLCTVDGQRHSVGDTKVPFCLQSCVKPLK
YAIAVNDLGTEYVHRYVGKEPSGLRFNKLFLNEDDKPHNPMVNAGAIVVTSLIKQGANNAEKFDYVM
QFMNKMAGNEYVGFSNATFQSERESGDRNFAIGYYLKEKKCFPEGTDMVAILDFYFQLCSIEVTCESA
SVMAATLANGGFCPIITGERVLSPEAVRNTLSLMHSCGMYDFSGQFAFHVGLPAKSGVAGGILLVVP
NVMGLMCWSPPLDKMGNSVKGIHFCHDLVSLCNFHNYDNLRHFAKKLDPRREGGDQRVKSVINLLF
AAYTGDVSALRRFALSGMDMEQRDYDSRTALHVAAAEGHVDVVKFLLEACKVNPFPKDRWNNTP
MDEALHLDTMMCLKFSKSIRSHTHRQRIPVMARRTERFIKTWMAYYNHSQLDPKNPISYLFNCGILK

Gase sequence alignment scores:

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	Weatherloach	220	2	Zebrafish	220	95
1	Weatherloach	220	3	Pufferfish	220	91
1	Weatherloach	220	4	Rat_Kidney	220	89
1	Weatherloach	220	5	Rat_Liver	220	77
1	Weatherloach	220	6	Human_Kidney	220	89
1	Weatherloach	220	7	Human_Liver	220	77
1	Weatherloach	220	8	Chicken	220	89
2	Zebrafish	220	3	Pufferfish	220	94
2	Zebrafish	220	4	Rat_Kidney	220	91
2	Zebrafish	220	5	Rat_Liver	220	80
2	Zebrafish	220	6	Human_Kidney	220	91
2	Zebrafish	220	7	Human_Liver	220	80
2	Zebrafish	220	8	Chicken	220	91
3	Pufferfish	220	4	Rat_Kidney	220	94
3	Pufferfish	220	5	Rat_Liver	220	79
3	Pufferfish	220	6	Human_Kidney	220	94
3	Pufferfish	220	7	Human_Liver	220	79
3	Pufferfish	220	8	Chicken	220	95
4	Rat_Kidney	220	5	Rat_Liver	220	81
4	Rat_Kidney	220	6	Human_Kidney	220	100
4	Rat_Kidney	220	7	Human_Liver	220	81
4	Rat_Kidney	220	8	Chicken	220	98
5	Rat_Liver	220	6	Human_Kidney	220	81
5	Rat_Liver	220	7	Human_Liver	220	99
5	Rat_Liver	220	8	Chicken	220	81
6	Human_Kidney	220	7	Human_Liver	220	81
6	Human_Kidney	220	8	Chicken	220	98
7	Human_Liver	220	8	Chicken	220	81

Weatherloach sequences for GDH:

>GDH Weatherloach (gene sequence)
TGTGTGAAATAACCAAAAATTACTCCGACAACGAATTGGAGAAAATTACAAGAAGGTTTACTATT
GAGCTGGCTAAAAAAGGCTTCATTGGGCCTGGGATTGACGTTCCAGCCCCAGACATGAGTACAG
GAGAAAGGGAAATGTCCTGGATTGCAGACACTTATGCAAACACTATGGGCCACCATGACATCAAT
GCTCATGCCTGTGTAACAGGGAAGCCCATCAGTCAGGGTGGCATTCATGGCCGTATATCTGCCAC
TGGTCGTGGCGTGTTTCATGGCATTGAAAACTTTGTCAATGAGGCTTCCTACATGAGCCAGCTGA
AAATGAATCCTGGCTTTGGGGACAAGACGTTTGTCATTCAGGGCTTTGGTAATGTGGGCCTACAC
TCCATGCGTTATCTACATCGCTTCGGAGCAAAGTGTGTTGGTGTGGGAGAAGTGGACGGAAGCA
TCTGGAACCCAAGCGGAATCGATCCCAAGGAGCTAGAGGACTACAAACTGGCCAACGGCACTAT
TGTCGGCTTCCCCAACGCCACTCCCTATGAAGGCAGCATCTTGGAAGCCGAATGCGATATCCTGA
TTCCTGCTGCAAGTGAAAAGCAGCTTACAAAGAAAAATGCCCACAATGTTAAAGCTAAGATCATC
GCTGAGGGAGCCAATGGCCCCACCACGCCAGAGGCAGACAGGATATTCCTAGAGAGAAATATCA
TGGTGATTCCTGATATGTACCTGAATGCTGGAGGTGTGACCGTCTCCTACTTCGAGTGGCTTAAG
AATCTTAATCATGTCAGCTATGGCAGACTGACCTTCAAATATGAAAGAGACTCAAACTACCATCTC
CTTATGTCTGTCCAGGAGAGTGTTGAAAG

>GDH Weatherloach (amino acid sequence)
CEITKNYSDNELEKITRRFTIELAKKGFIGPGIDVPAPDMSTGEREMSWIADTYANTMGHHDINAHAC
VTGKPISQGGIHGRISATGRGVFHGIENFVNEASYMSQLKMNPGFGDKTFVIQGFGNVGLHSMRYLH
RFGAKCVGVGEVDGSIWNPSGIDPKELEDYKLANGTIVGFPNATPYEGSILEAECDILIPAASEKQLTKK
NAHNVKAKIIAEGANGPTTPEADRIFLERNIMVIPDMYLNAGGVTVSYFEWLKNLNHVSYGRLTFKYE
RDSNYHLLMSVQESVERKFGKHRGPFPIVPTSDFQDR

Key Species full amino acid sequences:

>Zebrafish (Q6NZ29)
MYRYLGELVTRAAANSALASVCVDSALPASATLLRVRRYSEAVGEKDDDPNFFRMVEGFFDRGASIVE
DKLVHDLKTRETPEQKRHRVRGILKIIKPCNHVLSVSFPIKRDNGEWEMIEGYRAQHSQHRTPCKGGIR
YSTEVSVDEVKALASLMTYKCAVVDVPFGGAKAGVKINPKNYSDTELEKITRRFTIELAKKGFIGPGIDV
PAPDMSTGEREMSWIADTYANTMGHHDINAHACVTGKPISQGGIHGRISATGRGVFHGIENFVNEA
AYMSQLGLTPGFGDKTFVIQGFGNVGLHSMRYLHRYGAKCVGIGELDGSIWNPSGIDPKELEDYKLA
NGTIVGYPGATAYEGNILEAECDILIPAASEKQLTKKNANNIKAKIIAEGANGPTTPEADKIFLERNIMVI
PDMYLNAGGVTVSYFEWLKNLNHVSYGRLTFKYERDSNYHLLMSVQESLERKFGKHGGAIPIVPTSDF
QERISGASEKDIVHSGLAYTMERSARQIMRTANRYNLGLDLRTAAYVNAIEKVFKVYHEAGLTFT

>Rainbow_Trout (Q8JHY1)
MYRYFGELLTRGASSALASGCVESALPVTASLMRVRHYSEVVGEKDADDPNFFKMVEGFFDRGANIV
EDKLVEDLKNKETPVQKRHRVRGILKIIKPCNHILSVSFPIKRDNGEWEVIEGYRAQHSQHRTPCKGGIR
YSTEVSVDEVKALASLMTYKCAVVDVPFGGAKAGVKINVKNYTDNELEKITRRFTIELAKKGFIGPGIDV
PAPDMSTGEREMSWIADTYANTMGHHDINAHACVTGKPISQGGIHGRISATGRGVFHGIENFINEA

AYMSQLGLSPGFTDKTFVIQGFGNVGMHSMRYLHRFGAKCVGVGEMDGNIWNPNGIDPKELEDYK
LQHGTIVGFPNSTPYEGSILEADCDILIPAASEKQLTRNNAHKIKAKIIAEGANGPTTPDADKIFLERNIM
VIPDMYLNAGGVTVSYFEWLKNLNHVSYGRLTFKYERDSNYHLLMSVQESLERKFGKHGGAIPVVPTS
EFQARIAGASEKDIVHSGLAYTMERSARQIMRTANKYNLGLDLRTAAYVNAIEKVFKVYNEAGLTFT

>Atlantic_Salmon (C0PUN2)
ILSVSFPIKRDNGEWEVIEGYRAQHSQHRTPCKGGIRYSTEVSVDEVKALASLMTYKCAVVDVPFGGA
KAGVKINVKNYTDNELEKITRRFTIELAKKGFIGPGIDVPAPDMSTGEREMSWIADTYANTMGHHDIN
AHACVTGKPISQGGIHGRISATGRGVFHGIENFINEAAYMSQLGLSPGFTDKTFVIQGFGNVGMHSM
RYLHRFGAKCVGVGEMDGNIWNPKGIDPKELEDYKLQHGTIVGFPNSTPYEGSILEADCDILIPAASEK
QLTRNNAHKIKAKIIAEGANGPTTPDADKIFLERNIMVIPDMYLNAGGVTVSYFEWLKNLNHVSYGRL
TFKYERDSNYHLLMSVQESLERKFGKHGGAIPVVPTSEFQARIAGASEKDIVHSGLAYTMERSARQIM
RTANKYNLGLDLRTAAYVNAIEKVFKVYNEAGLTFT

>African_Clawed_Frog (Q6AZJ0)
MYRYIGELVSRGGGALASCTADSVLPLSAAPILRRYSQAVNGDDDPNFFKMVEGFFDRGAGIVEDKLV
EDLRTRETEEQKRLRVRGILRIIKPCNHVLSVSFPIKRDNGEWEVIEGYRAQHSQHRTPCKGGIRYSTEV
SVDEVKALASLMTYKCAVVDVPFGGAKAGVKINPRNFSDAELEKITRRFTIELAKKGFIGPGIDVPAPD
MSTGEREMSWIADTYANTIGHTDINAHACVTGKPISQGGIHGRISATGRGVFHGIENFINEASYMSQL
GMTPGFGDKTFVIQGFGNVGLHSMRYLHRFGAKCVGIGEIDGTIWNPNGIDPKELEDYKLQHGTIVG
FPKAQPYDGNILEADCDILIPAASEKQLTKSNAHKIKAKIIAEGANGPTTPEADKIFLERNIMVIPDLYLN
AGGVTVSYFEWLKNLNHVSYGRLTFKYERDSNYHLLMSVQESLERKFGKHGGAIPVVPTAEFQARISG
ASEKDIVHSGLAYTMERSARQIMRTAMKYNLGLDLRTAAYVNAIEKVFKVYNEAGLTFT

>Human (P00367)
MYRYLGEALLLSRAGPAALGSASADSAALLGWARGQPAAAPQPGLALAARRHYSEAVADREDDPNF
FKMVEGFFDRGASIVEDKLVEDLRTRESEEQKRNRVRGILRIIKPCNHVLSLSFPIRRDDGSWEVIEGYR
AQHSQHRTPCKGGIRYSTDVSVDEVKALASLMTYKCAVVDVPFGGAKAGVKINPKNYTDNELEKITRR
FTMELAKKGFIGPGIDVPAPDMSTGEREMSWIADTYASTIGHYDINAHACVTGKPISQGGIHGRISAT
GRGVFHGIENFINEASYMSILGMTPGFGDKTFVVQGFGNVGLHSMRYLHRFGAKCIAVGESDGSIW
NPDGIDPKELEDFKLQHGSILGFPKAKPYEGSILEADCDILIPAASEKQLTKSNAPRVKAKIIAEGANGPT
TPEADKIFLERNIMVIPDLYLNAGGVTVSYFEWLKNLNHVSYGRLTFKYERDSNYHLLMSVQESLERKF
GKHGGTIPIVPTAEFQDRISGASEKDIVHSGLAYTMERSARQIMRTAMKYNLGLDLRTAAYVNAIEKVF
KVYNEAGVTFT

>Mouse (P00367)
MYRRLGEALLLSRAGPAALGSAAADSAALLGWARGQPSAAPQPGLTPVARRHYSEAAADREDDPNF
FKMVEGFFDRGASIVEDKLVEDLKTRESEEQKRNRVRGILRIIKPCNHVLSLSFPIRRDDGSWEVIEGYR
AQHSQHRTPCKGGIRYSTDVSVDEVKALASLMTYKCAVVDVPFGGAKAGVKINPKNYTDNELEKITRR
FTMELAKKGFIGPGIDVPAPDMSTGEREMSWIADTYASTIGHYDINAHACVTGKPISQGGIHGRISAT
GRGVFHGIENFINEASYMSILGMTPGFGDKTFVVQGFGNVGLHSMRYLHRFGAKCVGVGESDGSIW
NPDGIDPKELEDFKLQHGSILGFPKAKVYEGSILEADCDILIPAASEKQLTKSNAPRVKAKIIAEGANGPT
TPEADKIFLERNIMVIPDLYLNAGGVTVSYFEWLKNLNHVSYGRLTFKYERDSNYHLLMSVQESLERKF
GKHGGTIPVVPTAEFQDRISGASEKDIVHSGLAYTMERSARQIMRTAMKYNLGLDLRTAAYVNAIEKV
FKVYNEAGVTFT

GDH sequence alignment scores:

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	Weatherloach	306	2	Zebrafish	306	93
1	Weatherloach	306	3	Rainbow_Trout	306	90
1	Weatherloach	306	4	Atlantic_Salmon	306	90
1	Weatherloach	306	5	African_Clawed_Frog	306	89
1	Weatherloach	306	6	Human	306	89
1	Weatherloach	306	7	Mouse	306	89
2	Zebrafish	306	3	Rainbow_Trout	306	91
2	Zebrafish	306	4	Atlantic_Salmon	306	91
2	Zebrafish	306	5	African_Clawed_Frog	306	90
2	Zebrafish	306	6	Human	306	87
2	Zebrafish	306	7	Mouse	306	88
3	Rainbow_Trout	306	4	Atlantic_Salmon	306	99
3	Rainbow_Trout	306	5	African_Clawed_Frog	306	92
3	Rainbow_Trout	306	6	Human	306	89
3	Rainbow_Trout	306	7	Mouse	306	89
4	Atlantic_Salmon	306	5	African_Clawed_Frog	306	91
4	Atlantic_Salmon	306	6	Human	306	89
4	Atlantic_Salmon	306	7	Mouse	306	89
5	African_Clawed_Frog	306	6	Human	306	91
5	African_Clawed_Frog	306	7	Mouse	306	91
6	Human	306	7	Mouse	306	98

Weatherloach sequences for GSase:

>GSase Weatherloach (gene sequence)
TCCTTTCCGCAAAGACCCCAACAAACTTGTCTTGTGTGAAGTTCTCAAATATAACCGGAAGCCAGC
TGAAACCAACCATCGTAAGACATGTAACAAGGTAATGGAAAAGGTAAGGGATCAAGTCCCTTGG
TTTGGCATGGAACAGGAGTACACKCTTTTGGGCACAGATGGACATCCATTTGGTTGGCCTTCAAA
CGGCTTYCCCGGTCCCCAAGGACCCTACTACTGTGGTGTTGGAGCAGACAAGGCCTATGGCAGAG
ACATCGTGGAGGCACATTATAGAGCTTGTCTGTATGCTGGTGTACAGATCTGTGGAACTAATGCT
GAAGTCATGCCAGCTCAGTGGGAATTCCAGGTTGGCCCTTGTGAAGGCATCAGCATGGGAGATC
ATTTGTGGGTCGCTCGTTTTATCTTGCACAGAGTATGTGAAGACTTTGGCGTGGTAGCTTCACTTG
ACCCCAAGCCGATTACTGGCAACTGGAACGGNCCTTGGCTGGG

>GSase Weatherloach (amino acid sequence)
PFRKDPNKLVLCEVLKYNRKPAETNHRKTCNKVMEKVRDQVPWFGMEQEYXLLGTDGHPFGWPSN
GXPGPQGPYYCGVGADKAYGRDIVEAHYRACLYAGVQICGTNAEVMPAQWEFQVGPCEGISMGD
HLWVARFILHRVCEDFGVVASLDPKPITGNWNXPWL

Key Species full amino acid sequences:

>Zebrafish (Q7T2P7)
MATSASSQLSKVVKQQYMELPQGDQVQAMYIWIDGTGEGLRCKTRTLDSEPKSIEDLPEWNFDGSS
TYQAEGSNSDMYLIPAAMFRDPFRKDPNKLVLCEVVKYNRKTAETNHRHTCKKIMEMVGHQSPWF
GMEQEYTILGTDGHPFGWPSNGFPGPQGPYYCGVGADKAYGRDIVEAHYRACLYAGVMICGTNAE
VMPAQWEFQVGPCEGIDMGDHLWVARFILHRVCEDFGVVASFDPKPIPGNWNGAGCHTNFSTKE
MREDGGLKCIEECIEKLGKRHNYHIRTYDPKGGLDNARRLTGHHETSNIHEFSAGVANRGASIRIPRAV
GQEKKGYFEDRRPSANCDPYAVTEALIRTCLLDEEGDETVDY

>Rainbow_Trout (Q8JI31)
MATSSSAELSKAVKQQYMDLPQGDKVQIMYVWIDGTGEGLRCKTRTLDSEPKCIEELPEWNFDGSST
YQSEGSNSDMYLIPSAMFRDPFRKDPNKLVLCEVLKYNRKPAETNLRLTCNKVMDMVENQVPWFG
MEQEYTILGTDGHPFGWPNNGFPGPQGPYYCGVGSDKAYGRDIVEAHYKACLYAGVMICGTNAEV
MPAQWEFQVGPCEGISMGDHLWAARFILHRVCEDFGVVASFDPKPIPGNWNGAGCHTNFSTKEM
REEGGLKAIEESIERLGKRHSYHIRAYDPKGGLDNARRLTGHHETSNIHEFSAGVANRGASIRIPRTVGQ
EKKGYFEDRRPSANCDPYAVTEAIIRTCLLSEEGDEPVNY

>Atlantic_Salmon (B5XCB2)
MATSSSAELSKAVKQQYMDLPQGDKVQIMYVWIDGTGEGLRCKTRTLDSEPKSIEELPEWNFDGSST
YQSEGSNSDMYLIPSAMFRDPFRKDPNKLVLCEVLKYNRKPAETNLRLTCNKVMDMVENQVPWFG
MEQEYTILGTDGHPFGWPNNGFPGPQGPYYCGVGSDKAYGRDIVEAHYRACLYAGVMICGTNAEV
MPAQWEFQVGPCEGISMGDHLWAARFILHRVCEDFGVVASFDPKPIPGNWNGAGCHTNFSTKEM
REEGGLKAIEESIERLGKRHSYHIRAYDPKGGLDNARRLTGHHETSNIHEFSAGVANRGASIRIPRTVGQ
EKKGYFEDRRPSANCDPYAVTEAIIRTCLLSEEGDEPVDY

>Pufferfish (Q8JIZ8)
MATSASASLSKAVKQQYMELPQGDKVQAMYIWIDGTGEGLRCKTRTLDSEPKSIEDLPEWNFDGSST
YQSEGSNSDMYLIPSAMFRDPFRKDPNKLVLCEVLKYNRKPTETNLRLTCKKVMDMVADQHPWFG
MEQEYTILGTDGHPFGWPSNGFPGPQGPYYCGVGADKAYGRDVVEAHYKACLYAGVQICGTNAEV
MPAQWEFQVGPCEGIDMGDHLWVARFILHRVCEDFGVVASFDPKPIPGNWNGAGCHTNFSTKEM
REDGGLKAIEDSIEKLGKRHSYHIRAYDPKGGLDNARRLTGRHETSNINEFSAGVANRGASIRIPRNVG
QEKKGYFEDRRPSANCDPYSVTEALIRTCLLNEEGDEPADY

>African_Clawed_Frog (Q6INY)
MATSDSAQLSKAIKQMYLELPQGDKVQAMYIWIDGTGEGLRCKTRTLDSEPKTIEDLPEWNFDGSST
HQSEGSNSDMYLIPVAMFRDPFRRDPNKLVLCEVLKYNRKTAETNLRHTCNQIMDMMGNEHPWF
GMEQEYTLLGMDGHPFGWPSNGFPGPQGPYYCGVGANKAYGRDIVEAHYRACLYAGVKIAGTNAE
VMPAQWEFQIGPCEGIDMGDHLWIARFILHRVCEDFGIIVSFDPKPITGNWNGAGCHTNFSTKSMR
EEEGLKHIEESIERLSKRHEYHIRMYDPRGGKDNARRLTGFHETSSIHEFSAGVANRGASIRIPRLVGQE
KKGYFEDRRPSANCDPYAVTEAIIRTCLLNETGDEPLEYKN

>Human (P15104)
MTTSASSHLNKGIKQVYMSLPQGEKVQAMYIWIDGTGEGLRCKTRTLDSEPKCVEELPEWNFDGSST
LQSEGSNSDMYLVPAAMFRDPFRKDPNKLVLCEVFKYNRRPAETNLRHTCKRIMDMVSNQHPWFG
MEQEYTLMGTDGHPFGWPSNGFPGPQGPYYCGVGADRAYGRDIVEAHYRACLYAGVKIAGTNAEV
MPAQWEFQIGPCEGISMGDHLWVARFILHRVCEDFGVIATFDPKPIPGNWNGAGCHTNFSTKAMR
EENGLKYIEEAIEKLSKRHQYHIRAYDPKGGLDNARRLTGFHETSNINDFSAGVANRSASIRIPRTVGQE
KKGYFEDRRPSANCDPFSVTEALIRTCLLNETGDEPFQYKN

>Rat (P09606)
MATSASSHLNKGIKQMYMNLPQGEKIQLMYIWVDGTGEGLRCKTRTLDCDPKCVEELPEWNFDGSS
TFQSEGSNSDMYLHPVAMFRDPFRRDPNKLVFCEVFKYNRKPAETNLRHSCKRIMDMVSSQHPWF
GMEQEYTLMGTDGHPFGWPSNGFPGPQGPYYCGVGADKAYGRDIVEAHYRACLYAGIKITGTNAE
VMPAQWEFQIGPCEGIRMGDHLWVARFILHRVCEDFGVIATFDPKPIPGNWNGAGCHTNFSTKAM
REENGLRCIEEAIDKLSKRHQYHIRAYDPKGGLDNARRLTGFHETSNINDFSAGVANRSASIRIPRIVGQ
EKKGYFEDRRPSANCDPYAVTEAIVRTCLLNETGDEPFQYKN

GSase sequence alignment scores:

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	Weatherloach	161	2	Zebrafish	161	90
1	Weatherloach	161	3	Rainbow_Trout	161	90
1	Weatherloach	161	4	Atlantic_Salmon	161	90
1	Weatherloach	161	5	Pufferfish	161	90
1	Weatherloach	161	6	African_Clawed_Frog	161	83
1	Weatherloach	161	7	Human	161	85
1	Weatherloach	161	8	Rat	161	83
2	Zebrafish	161	3	Rainbow_Trout	161	90
2	Zebrafish	161	4	Atlantic_Salmon	161	91
2	Zebrafish	161	5	Pufferfish	161	91
2	Zebrafish	161	6	African_Clawed_Frog	161	86
2	Zebrafish	161	7	Human	161	88
2	Zebrafish	161	8	Rat	161	87
3	Rainbow_Trout	161	4	Atlantic_Salmon	161	99
3	Rainbow_Trout	161	5	Pufferfish	161	93
3	Rainbow_Trout	161	6	African_Clawed_Frog	161	85
3	Rainbow_Trout	161	7	Human	161	87
3	Rainbow_Trout	161	8	Rat	161	85
4	Atlantic_Salmon	161	5	Pufferfish	161	92
4	Atlantic_Salmon	161	6	African_Clawed_Frog	161	85
4	Atlantic_Salmon	161	7	Human	161	88
4	Atlantic_Salmon	161	8	Rat	161	85
5	Pufferfish	161	6	African_Clawed_Frog	161	85
5	Pufferfish	161	7	Human	161	88
5	Pufferfish	161	8	Rat	161	86
6	African_Clawed_Frog	161	7	Human	161	88
6	African_Clawed_Frog	161	8	Rat	161	86
7	Human	161	8	Rat	161	94

ANNEX B – Protocols

3' RACE Protocol:

I. cDNA synthesis with adapter primer

1. Using the reagents provided in the Superscript III First Strand kit from Invitrogen add into a PCR tube:

   - 1µg total RNA
   - 1µL APT17 (adapter primer)
   - 1µL dNTPs
   - H_2O to a final volume of 10µL

2. Incubate at 70^{o}C for 10min and 4 ^{o}C for 5min.
3. Add to tube:

   - 2µL of 10x RT buffer
   - 4µL of 25mM $MgCl_2$
   - 2µL of 100mM DTT
   - 1µL RNase Out
   - 1µL Reverse Transcriptase

4. Incubate for 2 hours at 37^{o}C

II. Proceed with PCR with appropriate forward primer and AP reverse primer (if necessary use Platinum Taq). Resolve on a 1% agarose TAE gel.

III. Excise band(s) from gel and proceed with band extraction and purification using the illustra GFX PCR DNA and gel band purification kit.

IV. Polishing the Purified PCR Products (PCR-script Amp Cloning Kit)

1. Add in order into a PCR tube:

   - 10ul Purified PCR product
   - 1ul of 10mM dNTPmix
   - 1,3ul of 10x polishing buffer
   - 1ul of cloned *Pfu* DNA polymerase

2. Incubate for 30min at 72ºC on the thermalcycler
3. Store on ice or 4ºC for use

V. Ligating the Insert

1. Add in order to a PCR tube:

   - 1ul of pPCR-Script Amp SK(+) cloning vector
   - 1ul PCR-Script 10x Reaction Buffer
   - 0,5ul 10mM rATP
   - 2ul PCR Product
   - 1ul Srf I
   - 1 ul T4 DNA Ligase
   - 3,5ul H_20

2. Incubate 1h at room temperature
3. Incubate 10min at 65ºC
4. Place on ice or store at 4ºC

VI. Transformation

1. Thaw XL10-Gold Kan ultracompetent cells on ice. Meanwhile heat Hotblock temperature to 42ºC and confirm the temperature is exactly 42ºC with a thermometer. Place a 1ml aliquot of SOC broth on the block
2. Snip the tip off a 10 µL pipette tip. If cells have precipitated to the bottom of the tube (milky substance) before pipetting gently finger vortex and/or mix with the pipette in order to resuspend the cells
3. Gently mix 40ul of the cells into a chilled 1,5ml eppendorf tubes
4. Add 1,6ul of XL10 Gold β-mercaptoethanol and mix gently
5. Finger vortex and incubate on ice for 10min. Finger vortex every 2min.
6. Add 2ul of the ligation mix and finger vortex
7. Incubate on ice for 30min
8. Heat pulse the tubes 30sec at 42ºC (The temperature can NOT be over and the time should NOT exceed 30sec)
9. Incubate on ice 2min
10. Add 0,45ml of preheated SOC broth to each tube and incubate at 37ºC for 1h in the Hybridizer
11. Plate 200ul of the transformation reaction onto each plate
12. Incubate overnight at 37ºC

VII. Selection of colonies

1. Check overnight plates for the presence of blue and white colonies. Remove from incubator and place in fridge for 2h (makes color distinction easier)
2. Add 2ml of LB Broth (with ampicillin) to a sterile 15ml Falcon tube
3. With the tip of a 10ul pipette tip touch one of the white colonies (preferably choose isolated colonies and avoid colonies with a blue tint). Eject the tip into the broth. Label tubes and incubate in the hybridizer overnight.

Note: Be careful not to allow the cultures to grow for too much longer than 12hours

VIII. Verifying the clones

Check if tubes are milky if so:

1. Add 10ul of broth to 40ul of water
2. Heat to 99ºC for 5min
3. Spindown and procede with a PCR with primers which allow for identification of the correct insert (add 2,4ul of the sample to the PCR reaction)

IX. Obtain purified plasmids by using the Wizard plus sv minipreps DNA purification system from Promega and following the centrifugation protocol after obtaining a clear lysate.

X. Confirm correct predicted insert size by digesting the plasmids with EcoRI and resolving on gel and/or PCR with the m13 primer pair (and/or forward primer and reverse AP primer used for amplicon synthesis).

Medium preparation for cell cultures:

- Add 35g/L of Sigma LB Agar to water and mix so that the agar is in suspension
- Dissolve 20g/L of Sigma LB broth in water
- Autoclave both mediums for 2h at 120ºC
- Cool LB Agar to 60ºC in the incubator. LB Broth can be stored at room temp in the laminar flowhood and UV irradiated.

(IN LAMINAR FLOWHOOD)

- Add 100ul of Ampicillin [100mg/ml] for every 100ml of medium (both to LB Agar and Broth)
- Pour 20ml of LB Agar into each Petri dish
- Rest at room temperature with lid closed for 10-15min
- Remove the lid from Petri dishes and let sit for 2-3h at room temp (Can be stored upside down in refrigerator at this point)
- For each plate add 10ul of 0,1M IPTG and 20ul of 50mg/ml X-Gal to 100ul of SOC broth. Poor 100ul of mix onto each plate and spread evenly. (Do not forget to flame the spreader)
- Let absorb for 30min.

Plasmid Digestion:

- 0,5µL of EcoRI
- 1µL 10x buffer (provided with EcoRI)
- 1µL Purified Plasmid suspension
- 7,5µL Sigma H_2O

Incubate for 2 hours at 37^{o}C

PCR Protocol

For a Final Volume of 20µL mix for each tube:

Component	Conc. (Stock)	Conc. (Reac Mix)	V to add (µL)
H_2O Sigma	--	--	12,8
Reaction Buffer	10 x	1 x	2,0
$MgCl_2$	50 mM	2 mM	0,8
F primer	10 µM	0,4 µM	0,8
R primer	10 µM	0,4 µM	0,8
dNTPs	10 mM	0,8 µM	1,6
Taq Polymerase	2 U/µL	0,04 U/µL	0,4

- Add 19,2 µL of reaction Mix to each PCR tube
- Add 0,8 µL of sample to each tube

In thermalcycler program should read:

Step	Temp	Time
1	94°C	2 min
2	94°C	30 sec
3	X °C	30 sec
4	72°C	Y sec
5	72°C	5 min

X – Adequate temperature for annealing

Y – Time should vary accordingly (roughly 1min per 1kb)

iScript cDNA Synthesis Kit Protocol

For a Final Volume of 20µL mix for each tube:

Component	V to add (µL)
Nuclease-free Water	X
iScript Reaction Mix	4
iScript Rev. Transcriptase	1
RNA template	Y

X – Total volume of water to add is 20 µL- (5+Y µL)

Y – Total volume of RNA template corresponds to 1ug of RNA

In thermalcycler program should read:

Step	Temp	Time
1	25°C	5 min
2	42°C	30 min
3	85°C	5 min
4	4°C	Forever

Forever- storage temperature

ANNEX C – Gels and Buffers

TBE 2% Agarose Gel

2% Agarose Gel	
Agarose	0,6 g
10x TBE buffer	3 mL
MilliQ H_2O	Fill to 30 mL

Note: instead of filling to 30mL weigh 30g of water

Melt agarose in microwave (careful with pressure build up)
Let it cool to 65^{o}C (approximately 10-15min)
Add 0,3µL of EtBr (10mg/ml)

TAE 1% Agarose Gel

1% Agarose Gel	
Agarose	0,3 g
50x TAE buffer	0,6 mL
MilliQ H_2O	Fill to 30 mL

Note: instead of filling to 30mL weigh 30g of water

Melt agarose in microwave (careful with pressure build up)
Let it cool to 65^{o}C (approximately 10-15min)
Add 0,3µL of EtBr (10mg/ml)

1,2% Agarose/Formaldehyde Gel

1,2% FA Gel	
Agarose	0,36 g
10x MOPS buffer	3 mL
MilliQ H_2O	Fill to 30 mL

Note: instead of filling to 30mL weigh 30g of water

Melt agarose in microwave (careful with pressure build up)
Let it cool to 65^{o}C (approximately 10-15min)
Add 600µL of 37% formaldehyde (12,3M)
Add 0,3µL of EtBr (10mg/ml)

10x MOPS Buffer

For a Final Volume of 500mL

Reagent	MW (g/mol)	Concentration	Amount
MOPS	209,26	200 mM	20,926 g
Sodium Acetate	82,03	80 mM	3,2812 g
EDTA	292,25	10 mM	10 mL

Note: **TOXIC!!**

Dissolve MOPS and sodium acetate in DEPC water
Add EDTA and adjust the pH to 7.0 with NaOH
Filter sterilize (0,2µm)

10x TBE Buffer

For a Final Volume of 1L:

Reagent	MW (g/mol)	Amount
Tris Base	121,14	108 g
Boric Acid	61,8	55 g
Na_2EDTA	372,24	5 ml

Note: Na_2EDTA should be in a stock solution of 0,5M
Dissolve in 750mL MilliQ water and fill to correct volume after Autoclave

50x TAE Buffer

For a Final Volume of 1L:

Reagent	Stock	Amount
Tris Base	121,14 (g/mol)	242 g
Glacial Acetic Acid	100%	57,1 mL
Na_2EDTA	0,5 M	100 ml

Dissolve in 750mL MilliQ water and fill to correct volume after Autoclave